



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: A61K 31/385, 31/35	A1	(11) International Publication Number: WO 99/06040 (43) International Publication Date: 11 February 1999 (11.02.99)
(21) International Application Number: PCT/US98/16207 (22) International Filing Date: 4 August 1998 (04.08.98) (30) Priority Data: 60/055,433 4 August 1997 (04.08.97) US (71)(72) Applicants and Inventors: BERRY, Christopher, J. [NZ/TH]; 72/1 Soi Sailom, Phaholyothin Road (Soi 8), Samsennai, Phayathi, Bangkok 10400 (TH). FOLEY, John, L. [US/US]; 31 Campus Plaza Road, Hadley, MA 01035 (US). PACKER, Lester [US/US]; 64 Charles Hill Road, Orinda, CA 94563 (US). (74) Agents: BERMAN, Rod, S. et al.; Jeffer, Mangels, Butler & Marmaro LLP, 10th floor, 2121 Avenue of the Stars, Los Angeles, CA 90067-5010 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHOD OF TREATING DISEASE USING A TOCOTRIENOL AND ALPHA-LIPOIC ACID OR DERIVATIVES OR AN ESTER THEREOF (57) Abstract Antioxidant or combination of antioxidants which function particularly well in modulating the action of free radicals and in particular which function particularly well in regulating activation of a transcription factor, such as NF-kappa B and which can be non-toxically administered to a human or an animal are disclosed. In particular a method of treating diseases, including reduction of the incidence of lung cancers in tobacco smokers, using a tocotrienol and alpha-lipoic acid or tocotrienyl lipate or derivatives thereof is disclosed.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**METHOD OF TREATING DISEASE USING A TOCOTRIENOL
AND ALPHA-LIPOIC ACID OR DERIVATIVES OR AN ESTER THEREOF**

Field of the Invention

5 The present invention relates to a method of treating diseases, more specifically using a combination of a tocotrienol and alpha-lipoic acid or using a tocotrienyl lipate or using derivatives thereof. The present invention further relates, in particular to a method of reducing the occurrence of the excess incidence of cardiovascular disease and specific types of cancer in tobacco smokers.

10 **Background of the Invention**

Many diseases have been found to be caused in whole or in part by the detrimental action of free radicals. In order to treat such diseases the administration of a compound, referred to as an antioxidant, which counteracts the action of these free radicals, has been described. Such administration is disclosed by the following:

15 United States Patent No. 5,348,974 and United States Patent No. 5,217,992 disclose the use of tocotrienols to treat hypercholesterolemia, hyperlipidemia and thromboembolic disorders; United States Patent No. 5,376,361 discloses the use of tocotrienols and derivatives and tocopherols and tocopherol esters for topical administration to the skin to prevent and/or treat radiation-induced skin damage;

20 United States Patent No. 5,204,373 discloses the use of carba-tocotrienols as having superior metabolic stability over tocotrienols; United States Patent No. 4,603,142 discloses the use of a specific species of tocotrienol to lower cholesterol; United States Patent No. 5,334,612 discloses the treatment of retroviral diseases, such as HIV, using alpha-lipoic acid; United States Patent No. 5,084,481 discloses the

25 treatment of pain and inflammatory disorders, including diabetic neuropathy and arteriosclerosis, using dihydrolipoic acid.

Additionally, it has been found that many diseases caused in whole or in part by free radicals involve the expression of gene products which are under the control of the nuclear transcription factors known as NF-kappa B and AP-1. NF-kappa B and

30 AP-1 regulation in turn is modulated by the free radical status of a cell which can

activate translocation of NF-kappa B from the cell cytosol to the cell nucleus where it can bind to DNA and facilitate transcription of gene products involved in disease states. Antioxidants, by affecting the free radical status of the cell, can regulate the activation of NF-kappa B and AP-1.

5 A progressive deterioration in the antioxidant network characterizes the aging process. As a result of this deterioration, intracellular free radical levels rise. This in turn changes the regulation of the transcription of NF-kappa B and AP-1 related genes. More specifically, there is an age-associated alteration in nuclear factor kappa-B and AP-1 signal transduction pathways. See, e.g., Helenius et al., *Biochem.*
10 *J.*, 318 (Pt. 2) ():603-8 (Sept 1, 1996) (NF-kappa B); Wortwein et al., *J.Neurosci. Res.* 52(3):322-33 (May 1, 1998) (AP-1). AP-1 and NF-kappa B both show increased activities in older animals, whereas most other transcription factors decrease in DNA binding activities with age. Sierra et al., *J.Gerontol. A. Biol. Sci. Med. Sci.* 53(2):B102-B110 (Mar. 1998). Similar levels of NF-kappa B were found in the
15 cytosol of T-cells from both young and elderly donors. The precursor levels of NF-kappa B remain unaltered during aging.

 The age-associated decline is caused by altered regulation of the inhibitor I-kappa B, the redox-mediated activation step. Trebilcock et al., *Gerontology* 42(3):137-146 (1996). A functional antioxidant network assessed in terms of
20 glutathione levels is required for NF-kappa B activation. Ginn-Pease et al., *Biochem. Biophys. Res. Commun.* 226(3):695-702 (Sept. 24, 1996). Glutathione levels decline with age and disease.

 The transcription of gamma-glutamylcysteine synthetase is up-regulated by TNF-alpha. This up-regulation is attenuated by preincubation with high physiological
25 levels of glucose. Thus, high glucose levels impair the regulation of glutathione. See, e.g., Morales et al., *J. Biol. Chem.* 272(48):30371-9 (Nov. 28, 1997). Glutathione peroxidase overexpression in turn down-regulates thresholds of NF-kappa B activation by altering the isoform composition of I-kappa B. See, e.g., Suyang et al., *Mol. Cell. Biol.* 16(10):5444-9 (Oct. 1996); Kretz-Remy et al., *J.Cell. Biol.* 133(5):1083-93
30 (June 1996).

Cancers are among the diseases in which the action of free radicals has been implicated. Numerous other agents are known to cause various types of cancers. Among the various cancers, lung cancer is the most common cause of cancer deaths in men and women in the United States and worldwide. The two major histopathologies of lung cancer, adenocarcinoma (AC) and squamous carcinoma (SCC), affect smokers particularly. AC was very rare 30 years ago and not thought to be associated with tobacco use. However, the incidence of AC has risen 10 to 20 times in the last generation, and it now accounts for almost as many lung cancer cases as SCC. See, e.g., Stellman et al., *Prev. Med.* 26(4):451-6 (July-Aug. 1997); Wynder et al., *Environ. Health Perspect.*, 103 Suppl 8():143-8 (Nov. 1995).

Squamous carcinoma (SCC) is thought to be caused by polynuclear aromatic hydrocarbons (PAHs) in tars. It occurs in the entrances to the air passageways in the lung, whereas AC is promoted by gas phase components of cigarette smoke and occurs deep inside the lung tissue.

The huge increase in the rate of incidence of AC is thought to result from changes in cigarette manufacturing techniques. See Hoffman, J. *Toxicol. Environ. Health* 50(4):307-64 (March 1997). Since the 1950s, tobacco companies have altered the way in which cigarettes are formulated. Tar and nicotine yields have declined from a high of 38 milligrams of tar and 2.7 milligrams of nicotine in 1954 to 12 milligrams of tar and 0.95 milligrams of nicotine today. These reductions in tar content have been achieved by introducing filters and by adding nitrates to tobacco. Average nitrate levels have increased from 0.4% to just over 1%. These nitrates cause the cigarette to burn hotter, decreasing tar yields, but at the expense of increased generation of nitrogenous compounds, which are the gaseous carcinogens implicated in the rise of adenocarcinomas. By adding nitrates to tobacco, cigarette companies have reduced the tar yields which they are obliged to disclose but introduced a new hidden killer which apparently is responsible for a twenty-fold increase in the rate of adenocarcinomas.

PAHs, the main carcinogen in tobacco tar, are detoxified in two stages by human cells. First, cytochrome P450-related compounds break down the PAHs into

a form which can be solubilized by the enzyme glutathione S-transferase. Glutathione S-transferase bonds the PAH residues to glutathione, preparing them to be excreted in the water phase.

5 Glutathione is the major antioxidant system in the body, and is synthesized in
cells by enzymes from three dietary amino acids: glutamate, cysteine and glycine.
Glutathione-related enzymes are encoded by a superfamily of genes. See, e.g.,
Lafuente et al., *Cancer Lett.* 68 (1):49-54 (Jan. 15, 1993). These genes include GST
M1 and GST T1. Almost half of the population inherits less than optimal
10 glutathione-related genes. The GST M1 gene is deleted in 50% of the population and
the GST T1 gene in 16%. Hayes et al., *Crit. Rev. Biochem. Mol. Biol.* 20(6):445-600
(1995). Thus, as aging occurs, or as the functionality of the antioxidant network is
impaired by infection, carcinogens or metabolic stresses, or inherited suboptimal
genetic polymorphisms, the function of the transcription factors NF-kappa B and
AP-1, is attenuated. See, e.g., Pinkus et al., *J. Biol. Chem.* 271(23):13422-9 (June
15 7, 1996). It has been suggested that these genetic abnormalities are major
determinants of susceptibility to degenerative diseases such as cancers, for example
tobacco-related lung cancers, and many studies have found elevated risks. Further,
as the normal production of cytokines is disturbed, there is dysregulation of the
hypothalamic/pituitary/adrenal (HPA) axis, resulting in hypertension, diabetes, arterial
20 sclerosis and heart failure. Inherited variations in the genes which regulate
antioxidant enzyme transcription exacerbate these conditions. Subjects inheriting
polymorphisms of the cytochrome P450 and glutathione transferase gene families are
at elevated risk of degenerative disease.

 Recently researchers at Ninewells Hospital and Medical School in Dundee,
25 Scotland, bred a strain of mice in which the gene that encodes glutathione
S-transferase was knocked out. See *Proc. Nat'l Acad. Sci. USA* 95(9):5275-80 (April
28, 1998). This genetic structure mimics the abnormality found in 46% of
Caucasians. These mice were shown to be three times more likely to develop
carcinomas as a result of PAH exposure.

Early human studies (e.g., Grinberg-Funes et al., *Carcinogenesis* 15(11):2449-54 (Nov. 1994) found elevated risks of lung cancer in humans who inherit the structure, but not all of these studies have shown high increased risk of carcinoma development. Recently, several studies have reported that weaknesses in the glutathione-related genetic mechanisms characterize early-onset lung cancer. See, e.g., el-Zein et al, *Mutat. Res.* 381(2):189-200 (Nov. 28, 1997); Bouchardy et al., *Cancer Res.* 56(2):251-3 (Jan. 15, 1996); Kihara et al., *Carcinogenesis* 16(10):2331-6 (Oct. 1995); Anttila et al., *Cancer Res.* 55(15):3305-9 (Aug. 1, 1995); Nakajima et al., *Carcinogenesis* 16(4):707-11 (Apr. 1995); Kihara et al., *Carcinogenesis* 15(2):415-8 (Feb. 1994); Kawajiri et al., *Princess Takamatsu Symp.* 21(): 55-61 (1990).

These studies show an interplay between total pack-years of smoking, glutathione S-transferase knockout and lung cancer. In people with compromised glutathione S-transferase genes, lung cancer risk elevates dramatically with increased total exposure to tobacco smoke. It appears that susceptibility to lung cancer results from the interplay between the inherited capacity of the detoxification mechanisms and lifetime exposure to tobacco smoke carcinogens. Prolonged smoking exhausts the detoxification system. In people inheriting a weak genetic detoxification structure this occurs more quickly.

Furthermore, cigarette smokers are at elevated risk of stroke, hypertension and cardiovascular disease. Approximately half of the premature death among smokers is from heart attacks and strokes. Cigarette smokers have elevated cortisol levels (see, e.g., Pomerleau et al., *Pharmacol. Biochem. Behav.* 36(1):211-3 (May 1990)), and whole blood from smokers has been found to have a 38% higher TNF production after lipopolysaccharide stimulation. Tappia et al., *Clin. Sci. (Colch.)* 88(4):485-9 (April 1995).

Often the mechanisms by which cigarette smoking exacerbates these conditions are related to dysregulation of the HPA axis, which modulates the interplay between the hormones which regulate glucose metabolism (insulin), and those that counter-regulate glucose metabolism (glucocorticoids). Epinephrine and

norepinephrine mediate short-term adjustments, whereas cortisol influences longer term cyclic responses.

5 The apparent insulin resistance and hypertension which characterize cigarette smokers, Syndrome X, adult onset diabetes and Cushing's Syndrome, share a common antecedent in disturbances to the HPA axis. Insulin resistance can be considered as dysregulation of and/or an increased tissue sensitivity to glucocorticoids. Thioredoxin, a redox-regulating cellular cofactor that modulates cellular glucocorticoid responsiveness, influences tissue sensitivity to glucocorticoids. See, e.g., Makino et al., J. Clin. Invest. 98(11):2469-77 (Dec. 1, 1996).

10 The HPA axis is a complex open system, modulated by counter-regulatory hormones, by inhibitory feedback loops and by cytokines. Cytokines modulate glucocorticoid hormones and are modulated by them. Since cellular production of cytokines is controlled by transcription factors, principally NF-kappa B, and NF-kappa B activation is controlled by cellular redox status, antioxidants modulate the HPA axis.

15 The interaction between glucocorticoids and insulin is mediated through thresholds of NF-kappa B expression. NF-kappa B and the glucocorticoid receptor physically interact, resulting in the repression of NF-kappa B transactivation. Glucocorticoids inhibit cytokine gene transcription through receptor cross-coupling. The activated glucocorticoid receptor can bind to and inactivate AP-1 and NF-kappa B.

20 Glucocorticoids strongly downregulate TNF-alpha. Pretreatment of cells with glucocorticoids modulates NF-kappa B activation, TNF-alpha and serum glucose. Administration of glucocorticoids alters hormonal and cytokine response to lipopolysaccharide in humans. Elevated TNF and cortisol levels have been found to exaggerate S-TNF-R responses for up to 144 hours after infusion, and in some tissue glucocorticoids upregulate the transcription of I-kappa B isoforms which bind to NF-kappa B in the cytosol and modulate NF-kappa B mediated gene transcription. Glucocorticoids thus are the link between insulin resistance and hypertension.

25 The HPA system, as a whole, is in dynamic semi-stable equilibrium and pivots

around set points analogous to thermostats. At birth, these set points are predetermined by the environment of the womb, when the fetus is exposed to the maternal regulatory system. Consequently, it has been demonstrated that low birth weight children have higher cortisol levels as adults, much higher incidence of adult-onset diabetes, and hypertension, stroke and heart disease as adults. Subsequently, contemporary stresses such as nocturnal hypoglycemia, disrupted sleep patterns, psychological stress, cigarette smoke, environmental toxins or aging, can disrupt the setpoints and influence HPA function by affecting NF-kappa B activation thresholds.

The onset of degenerative disease in middle age is frequently foreshadowed by cumulative changes in HPA responses consequent to exhaustion of the antioxidant network measured by decreasing glutathione levels, increasing TNF alpha levels, and a lowered threshold of NF-kappa B activation, increasing blood pressure, impairment of the diurnal regulation of cortisol, increased tissue sensitivity to glucocorticoids and apparent insulin resistance.

Excess cortisol has profound effects on the metabolism of glucose and protein. It increases gluconeogenesis by increasing glycogenolysis in muscle to provide lactate as a gluconeogenic precursor. It increases lipolysis in the periphery to provide glycerol as a gluconeogenic precursor. Since cortisol has anti-insulin effects, it decreases the uptake of glucose into muscle, adipose, lymphoid and fibroblast tissues. Excess cortisol also breaks down muscle proteins to provide gluconeogenic precursors to the liver. Disregulation of these effects lead to hyperglycemia and a characteristic pattern of central obesity.

The programming of the HPA axis has been shown to influence body weight, blood pressure body fat disposition patterns, insulin resistance and depression. Diabetics have significantly greater 24-hour urinary-free cortisol output which increases with duration of diabetes and degree of diabetic complications. Diabetic neuropathy is associated with specific and persistent increases in HPA axis activity. Cortisol levels are elevated in symptomatic polyneuropathy.

Cytokines whose expression is controlled by NF-kappa B, particularly TNF-alpha, the cytokine implicated in insulin resistance, modulate the HPA axis and certain

of the metabolic consequences of cortisol elevation are modulated by redox sensitive steps.

Glucocorticoids also have mineralocorticoid activities. Excess glucocorticoids may spill over onto aldosterone receptors to increase renal Na^+ absorption and increase renal K^+ and H^+ excretion, retaining water in the ECF, and elevating blood pressure. Also, glucocorticoids synergize catecholamines, increasing stroke volume and cardiac output which can also cause hypertension.

Cortisol levels rise in response to nicotine in smokers and are permanently elevated by cortisol-secreting tumors in Cushing's Syndrome, while in adult onset diabetes the diurnal cycle of cortisol secretion is disturbed and cortisol responses to TNF are exaggerated. Inappropriately elevated cortisol levels affect renin/angiotensin receptors inducing sodium retention and hypertension, counter-regulate insulin and attenuate insulin-mediated uptake of glucose by GLUT-4 receptors in muscle tissue. Cigarette smoke condensate activates NF-kappa B, directly compounding the disregulation. See Shen et al., Am.J.Physiol. 270(5 Pt.2):H1624-33 (May 1996).

Thus, the disruption of normal HPA axis function caused when nicotine raises cortisol levels and ROS overwhelm the antioxidant network contributes substantially to the elevated cardiovascular risk of smokers. One of the consequences of nicotine elevating cortisol levels in smokers is that cortisol, which normally responds to low blood glucose levels in order to protect the brain from hypoglycemia, is instead elevated by a false nicotine-mediated signal and insulin down-regulated when blood glucose levels are still high, resulting in the area under the curve (AUC) of blood glucose rising and further impairing the transcription of protective antioxidant enzymes. Urata et al., J. Biol. Chem. 271(25):15146-52 (June 21, 1996). Consequently, glycation and the formation of advanced glycation end products (AGEs) is elevated, raising cardiovascular risks by mechanisms similar to those by which the cardiovascular risks of diabetics are raised. AGE and AGE receptor (RAGE) interactions activate NF-kappa B, as does glycated LDL, and activated NF-kappa B is found in arteriosclerotic lesions.

AGE-apolipoprotein B and serum AGE levels in cigarette smokers have been found to be significantly higher than those in non-smokers. See Cerami et al., "Tobacco Smoke is a Source of Toxic Reactive Glycation Products," The Picower Institute for Medical Research, Manhasset, NY 11030 USA (1997). Cigarette smoke is a source of reactive AGE compounds. Id. It is therefore not surprising that diabetics who smoke have 2-4 times the incidence of cardiovascular disease than non-smoking diabetics.

The HPA is an open, adaptive system whose components interact in complex, self-regulating redundant patterns. The effects of isolated components can be paradoxical and shifted in time. For example, severely elevated cortisol levels can produce depression or euphoria, and normal responses to stimuli can be inverted or attenuated in diabetics and hypertensives or after prolonged oxidative stress.

The cellular processes which control the gene transcription of cytokines depend on the integrity of the antioxidant signaling network. In many degenerative conditions, including the tobacco-related diseases, it is the attenuation of the antioxidant signaling network by increasing reactive oxygen species' stress loads that trigger disease onset. Redox sensitive pathways modulate the HPA axis directly, by controlling the transcription of cytokine messengers, and indirectly by ameliorating the consequences of HPA dysfunction by reducing glycation damage. Thus, strengthening and reinforcing this network can delay the onset of degenerative diseases.

Accordingly, a need exists for an antioxidant or combination of antioxidants which function particularly well in maintaining the integrity of the HPA axis and protecting against the elevated cardiovascular risk reflected in impaired glucose tolerance, increased formation of advanced glycation end products and hypertension.

A need also exists for an antioxidant or a combination of antioxidants which function particularly well in modulating the action of free radicals and in particular which function particularly well in regulating activation of NF-kappa B and which can be non-toxically administered to a human or an animal.

A need also exists for a method of treating diseases, such as cancer, in which the action of free radicals has been implicated.

There is also a need for a method of reducing the occurrence of cancers in human patients who are especially susceptible to such occurrence. In particular, a need exists for a method of reducing the occurrence of lung cancer in human patients who smoke tobacco products and who are predisposed to lung cancer by inherited polymorphisms in cytochrome P450 1A1 and glutathione S-transferase M, such as those described, e.g., in Lafuente et al., Cancer Lett. 68(1):49-54 (Jan. 15, 1993); Kihara et al., Carcinogenesis 16(10):2331-6 (Oct. 1995); Grinberg-Funes et al., Carcinogenesis 15(11):2449-54 (Nov. 1994); and Bouchardy et al., Cancer Res. 56(2):251-3 (Jan. 15, 1996).

Summary of the Preferred Embodiments

In accordance with one aspect of the present invention, there are provided methods of treating and preventing a disease selected from the group consisting of cancer, arterial sclerosis, abnormal platelet aggregation, hypertension, congestive lung disease, diabetes, viral infection, septic shock, graft versus host disease, cystic fibrosis, autoimmune disease, neurodegenerative disease, asthma, gout, kidney failure, Parkinson's disease, multiple sclerosis, and Alzheimer's disease. The methods comprise the step of administering to a human or an animal, preferably a mammal, in need of such treatment a composition including an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to reduce or prevent a symptom of such disease.

In accordance with another aspect of the present invention, there is provided a method of regulating cellular apoptosis. The method comprises the step of administering to a human or an animal a composition including an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to regulate cellular apoptosis.

According to another aspect of the present invention there are provided methods of treating and preventing a disease caused at least in part by the effect of reactive oxygen species on NF-kappa B. The method comprise the step of administering to a human or an animal, preferably a mammal, in need of such treatment a composition including an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof, thereby regulating NF-kappa B.

In a preferred embodiment, the composition is administered in an amount effective to reduce transcription of a gene product controlled by NF-kappa B by between about 5% and about 99%.

Preferably the gene product is selected from the group consisting of a cytokine, a cytokine receptor, a cell adhesion molecule, a viral protein, a growth factor, a growth factor receptor, an immunoreceptor, a transcription factor, an oncogene and nitric acid synthase.

In accordance with another aspect of the present invention, there is provided a method of regulating cellular NO_x metabolism comprising the step of administering to a human or an animal a composition including an ingredient selected from the group consisting of a gamma-tocotrienol, a gamma-tocotrienyl lipoate, a combination of a gamma-tocotrienol with alpha-lipoic acid, derivatives thereof, such as other isomers of vitamin E that are "free" at the ortho position on the phenolic moiety, and combinations thereof.

In accordance with another aspect of the present invention there is provided a method of improving the regulation of the HPA axis-mediated interplay between cortisol and insulin and of protecting the cardiovascular system from the sequelae of its disturbance, hypertension, diminished HDLC levels, and increased advanced glycation end products.

In a first preferred embodiment, the method of regulating cellular NO_x metabolism comprises regulating nitric oxide synthase activity.

In a second preferred embodiment, the method of regulating cellular NO_x metabolism comprises decreasing the formation of peroxynitrites.

In yet another aspect of the present invention there is provided a composition including an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof, and a pharmaceutically acceptable carrier. Preferably the composition is formulated in an amount from greater than about 1000 mg to about 2500 mg.

Preferably the foregoing derivatives are selected from the group consisting of linaloyl lipoate, farnesyl lipoate, geranyl lipoate, nerol lipoate, gamma-hydroxybutyrate ester of alpha lipoic acid, gamma-hydroxybutyrate ether of alpha lipoic acid, cysteine ester of tocopherol, cysteine ester of tocotrienol, succinate ester of tocotrienol, and succinate ether of tocotrienol. The derivatives are preferably administered in an amount from about 25 mg to about 1000 mg.

In accordance with still another aspect of the present invention, there is provided a method of reducing the occurrence of a disease selected from the group consisting of adenocarcinoma and squamous cell carcinoma in a human who smokes tobacco products and is predisposed to lung cancer by at least one inherited polymorphism selected from the group consisting of a cytochrome P450 1A1 polymorphism and a glutathione S-transferase M1 polymorphism, which comprises the step of administering to the human a composition including an effective detoxifying amount of an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof.

In accordance with still another aspect of the present invention, there is provided a method of reducing the occurrence of a disease selected from the group consisting of adenocarcinoma and squamous cell carcinoma in a human who smokes tobacco products and is predisposed to lung cancer by at least one inherited polymorphism selected from the group consisting of a cytochrome P450 1A1 polymorphism and a glutathione S-transferase M1 polymorphism which comprises the step of administering to the human a composition including an effective amount of a component that promotes the intracellular conversion of cystine to cysteine.

In a preferred embodiment, the composition further includes an effective amount of a components that reduces the intracellular formation of at least one nitrosamine.

5 Other objects, features and advantages of the present invention will become apparent to those skilled in the art from the following detailed description. It is to be understood, however, that the detailed description and specific example, while indicating preferred embodiments of the present invention, are given by way of illustration and not limitation. Many changes and modifications within the scope of the present invention can be made without departing from the spirit thereof, and the
10 invention includes all such modifications.

Detailed Description of the Preferred Embodiments

It has been discovered that tocotrienyl lipoate or a combination of a tocotrienol and alpha-lipoic acid act synergistically as compared to a tocotrienol alone or alpha-lipoic acid alone in reducing or blocking inflammatory responses and in regulating the
15 activity of transcription factors sensitive to a cell redox state, such as the DNA transcription factor NF-kappa B.

NF-kappa B is part of a family of Rel transcription factors that share common characteristics. They consist of hetero- or homodimeric proteins in association with an inhibitory protein family, I-kappa B. It is believed that one mechanism by which
20 NF-kappa B is activated is by phosphorylation and dissociation of I-kappa B from Rel protein dimers, followed by I-kappa B's proteolytic degradation, which allows the dimeric DNA-binding protein NF-kappa B to enter the nucleus and regulate gene transcription. A host of genes have been shown to be modulated by NF-kappa B, including genes for cytokines and growth factors, immunoreceptors, adhesion
25 molecules, acute-phase proteins, transcription factors and regulators, NO-synthase, and viral genes. A general description of the NF-kappa B transcriptional regulation system can be found in Bauerele and Baltimore, *Cell*, Volume 87, 13-20 (1996), incorporated herein by reference.

Oxygen free radicals and nitrogen monoxide and their by-products that are
30 capable of causing oxidative damage are collectively referred to as active or reactive

oxygen species ("ROS") and reactive nitrogen species ("RNS"). These compounds are also known as "free radicals." These ROS can activate NF-kappa B and it is believed that ROS are the final common signal for a number of stimuli that activate NF-kappa B. Sen and Packer, *The FASEB Journal*, Vol. 10, 709-720 (1996). The administration of antioxidants which can modulate the ROS status of a cell should therefor be useful in regulating NF-kappa B activation. The activation of NF-kappa B is believed to be involved, at least in part, in the causation or progression of a number of disease states. Packer et al., *Advances in Pharmacology*, Vol. 38, 79-101 (1997).

For example, acquired immunodeficiency syndrome results from infection with a human immunodeficiency virus (HIV-1 or HIV-2), which eventually destroys a specific subset (CD4+) of helper T-lymphocytes, so that the patient ultimately yields to opportunistic infection and certain neoplasms. The long-terminal repeat (LTR) region of HIV-1 proviral DNA contains two binding sites for NF-kappa B, which activate transcription by binding to the sequence 5'-GGGACTTTCC-3' in the kappa enhancer where it interacts with the transcription apparatus. In this case, and for other viruses as well, the virus usurps normal cellular machinery in order to transcribe its own DNA.

By blocking activation of NF-kappa B, interference with the viral reproductive cycle can be facilitated.

Oxidants appear to be involved in two steps of the atherosclerotic process. Low density lipoprotein ("LDL") particles enter the artery wall and some remain. In this environment the LDL becomes mildly oxidized, possibly by ROS or RNS released by vascular cells. The mildly oxidized LDL contains a component or components, believed to be oxidized phospholipid, which causes the endothelium to secrete molecules that result in the recruitment of monocytes and their differentiation to macrophages. Further oxidation of the LDL results in its uptake by macrophages, their conversion to foam cells, and deposition in the vascular wall as part of fatty-streak lesions. Maziere et al., *Biochem. and Molec. Bio. Intl.*, Vol. 39(6) pp. 1201-1207 (1996).

NF-kappa B appears to be a central transcription factor in the expression of a number of the genes induced by the early oxidation step in atherosclerosis, including serum amyloid A and macrophage colony-stimulating factor. When C57BL/6J mice and C3H/HeJ mice, which are susceptible and not susceptible to formation of aortic fatty streaks, respectively, are fed an atherogenic diet, only the C57BL/6J mice show activation of NF-kappa B.

There is a further acceleration of arterial sclerosis in diabetes and in cigarette smokers, which is consequent to prolonged elevation of blood glucose levels. This is hypothesized to be due to advanced glycosylation end products (AGE) interacting with their endothelial receptor to induce the expression of vascular cell adhesion molecule-1 (VCAM-1), an early feature in the pathogenesis of atherosclerosis. In cultured human endothelial cells, exposure to AGEs induced expression of VCAM-1 associated with increased levels of VCAM-1 transcripts; electrophoretic mobility shift assays indicated that there was also induction of specific DNA-binding activity for NF-kappa B in the VCAM-1 promoter, which was blocked by N-acetyl cysteine ("NAC"). Other studies also indicate that the induction of expression of VCAM-1 in response to a variety of signals is mediated by an NF-kappa B-like DNA-binding protein and is blocked by a variety of antioxidants, including pyrrolidine dithiocarbamate and NAC.

Accelerated arterial sclerosis in diabetes and cigarette smokers may be due to the presence of larger amounts of AGE, a greater degree of interaction of these with the receptor for AGE (AGE-RAGE interaction), activation of NF-kappa B, expression of VCAM-1, and priming of the diabetic vasculature for enhanced interaction with circulating monocytes.

By interfering with the activation of NF-kappa B, these diseases, and other diseases which are believed to be caused at least in part by the effect of reactive oxygen species on NF-kappa B (for example by NF-kappa B activation of gene transcription) can be treated. The following diseases in addition to the above-listed are believed to be so caused: cancer, cancer metastasis, abnormal platelet aggregation, hypertension, congestive lung disease, viral infection, such as HIV/AIDS, septic shock, graft versus host disease, cystic fibrosis, autoimmune disease,

neurodegenerative disease, asthma, gout, kidney failure, Parkinson's disease, multiple sclerosis, and Alzheimer's disease, as well as cellular apoptosis. With respect to cellular apoptosis, regulation of NF-kappa B with the compositions of the present invention can cause or inhibit apoptosis, depending upon the particular cell type and the redox state of the cell, thereby regulating cellular apoptosis. To the extent that an animal is susceptible to disease states caused by NF-kappa B activation, such animal may be similarly treated.

A disease may be considered treated by the inventive methods if a symptom of the disease is improved to the extent that an improvement in the symptom is measurable by a standard test or perceptible to a human with the disease. For example, an increase in CD4+ T-cell levels as measured by standard assay, in a patient with AIDS. See, A Manual of Laboratory and Diagnostic Tests, Fifth Ed. Francis Fishbach (1996), incorporated herein by reference.

A disease may be considered prevented by the inventive methods if a disregulation or imbalance leading to the condition is corrected.

While not being bound by a particular theory, it is believed that the synergistic effect of tocotrienols and alpha-lipoic acid is the result of the phytyl side chain of tocotrienols which provides enhanced lipophilic mobility and intermembrane transferability to the tocotrienols. Also, tocotrienols are distributed evenly throughout the biomembrane whereas alpha tocopherol is clumped in relatively immobile clusters. Accordingly, tocotrienols are more readily available to more cellular compartments to interact with free radicals in those compartments. Further, after oxidation of a tocotrienol by its interaction with a free radical the tocotrienol may be more readily reduced by alpha-lipoic acid and thereby able to again interact with a free radical than less mobile antioxidants. The importance of this molecular mobility in ultimately regulating NF-kappa B activity was not heretofore recognized. Further, while some antioxidants can be administered directly to cell cultures in vitro in an amount that will provide a concentration sufficient to affect NF-kappa B activity, the compositions of the present invention, particularly tocotrienols and alpha lipoic acid, unlike the afore-mentioned antioxidants, can be preferably orally administered or administered

by another route to a human or an animal in an amount to provide a concentration in vivo sufficient to modulate NF-kappa B activity.

5 The regulation of cellular nitrogen monoxide ("NO") and nitrogen dioxide ("NO₂") metabolism ("NO_x") is important with respect to carcinogenesis, particularly carcinogenesis caused by exposure to environmental air pollutants. NO₂ can form peroxynitrites which are potent free radicals and can damage cells by inducing lipid peroxidation and protein oxidation. Peroxynitrites can also nitrosate amines; nitrosated amines can cause DNA mutations, which can lead to carcinogenesis. NO_x radicals can also activate NF-kappa B which in turn can activate transcription of
10 inducible nitrogen monoxide synthase.

Inducible nitrogen monoxide synthase is long lasting, Ca²⁺ independent and glucocorticoid sensitive. Nitrogen monoxide synthase generates NO leading to a positive feedback cycle of inducible nitrogen monoxide synthase production. In contrast, constitutive nitrogen monoxide synthase activity is short lasting, having one
15 sixth to one tenth the activity of inducible nitrogen monoxide synthase, is Ca²⁺ dependent and is hormone insensitive. It has been found that gamma-tocotrienols due to the absence of a methyl group at the C-5 position can form a C-nitroso aromatic compound which thereby decreases the production of detrimental peroxynitrites, thereby regulating "NO_x" metabolism. This effect can be augmented
20 by administering or esterifying alpha-lipoic acid with the gamma-tocotrienol, due to the ability of the alpha-lipoic to regenerate oxidized tocotrienol radicals to tocotrienols. Gamma-tocotrienols are believed to preferentially operate by the same mechanisms as set forth above. Therefore administration of gamma-tocotrienols can reduce free radical induced DNA mutagenesis and resulting carcinogenesis.

25 It has further been discovered that the administration of alpha lipoic acid and tocotrienols in a compound or mixture according to the present invention is effective in treating cancers, in particular lung cancer, both therapeutically and prophylactically. That is, it has been discovered that such administration is effective in protecting against both the AC and SCC forms of lung cancer associated with
30 cigarette smoking. Such compositions, in particular gamma-tocotrienol lipoate, are

especially effective in maintaining glutathione-related detoxification mechanisms in cigarette smokers who have inherited sub-optimal combinations of the cytochrome P450 and glutathione gene superfamily. Further, the gamma-isomer of tocotrienol appears to offer additional protection against nitrosamine-induced adenocarcinomas.

5 Han et al., Biofactors 6(3):321-38 (1997) have shown that the rate limiting step in the cellular synthesis of glutathione is the availability of cysteine in the cytosol. Glutathione is the most important antioxidant/detoxification pathway in the human body. Depressed glutathione levels are an indicator of mortality in many disease models. Yet glutathione levels are notoriously difficult to boost. Packer et al.
10 showed that administration of alpha-lipoic acid to cell cultures elevated glutathione levels by 30% by splitting cystine to cysteine, thereby facilitating the entry of cysteine into the cell by a channel that is 10 times faster than the cystine channel. Since the final step in the detoxification of PAHs requires the enzyme glutathione S-transferase to join glutathione to the toxicant, boosting or restoring intracellular
15 glutathione levels indirectly through alpha-lipoic administration, may help protect those smokers with an inherited attenuation of the glutathione system which detoxifies PAHs, thereby reducing their vulnerability to squamous carcinomas. Since the epidemiological pattern indicates that squamous carcinomas occur after the detoxification system has been exhausted by long-term exposure to cigarette smoke,
20 it follows that administration of alpha-lipoic may have a very significant role in boosting glutathione-related detoxification mechanisms thereby postponing or preventing the incidence of squamous carcinomas.

 Tocotrienols have been reported to have various anti-cancer effects including the modulation of glutathione S-transferase levels in animal cancer models. In
25 particular, gamma-tocotrienol is 40 to 60 times more readily recycled in membranes than alpha-tocopherol, and the gamma-isomers of tocopherols and tocotrienols appear to function to detoxify nitrogen oxides present in cigarette smoke by protecting against the formation of carcinogenic nitrosamines. Furthermore, alpha-lipoic acid appears to recycle tocotrienols. The effects of tocotrienols and alpha-lipoic have
30 been shown to be synergized when co-administered.

Remarkably, each of these compounds synergizes the other within the domain of its respective protective action. The addition of tocotrienols to alpha-lipoic acid potentiates the modulation by alpha-lipoic acid of the glutathione-related pathways and reinforces the effectiveness of cytochrome P450, while the addition of
5 alpha-lipoic acid to tocotrienols facilitates the systemic recycling of tocotrienols, thereby facilitating detoxification of nitrogen metabolites. The compensatory modulation of gene expression achieved by this pairing is unique and previously unrecognized.

Preferably, an effective amount of the inventive composition for use in
10 preventing the occurrence of lung cancers as discussed herein is about 5 mg to about 2500 mg, more preferably about 100 mg to about 1250 mg. Preferably the molar ratio of alpha-lipoic acid to tocotrienol is about 1:100 to about 2:1. The R-form of alpha-lipoic acid is particularly potent. Advantageously about 2-10 mg of the R-form (2-10 times the usual dietary intake) are employed to synergize with tocotrienol.
15 Naturally occurring R-alpha lipoic acid is frequently found in lipoamide form.

Desirably, the inventive compositions described herein are administered in divided doses 30-45 minutes prior to meals. However, other modes of administration of the inventive compositions will also be readily apparent to those skilled in the art. For example, the inventive compositions can be incorporated into a functional food
20 for consumption by a patient.

According to the invention, a reduction in the *in vivo* glutathione level of about 5-30% is realized, resulting in effective reduction in the occurrence of AC and SCC in tobacco smokers who are predisposed to lung cancer by virtue of an inherited polymorphism in cytochrome P450 1A1 and/or glutathione S-transferase M1.

25 The inventive compositions can also include additional antioxidants and/or nutraceuticals, such as vitamin C, vitamin B₆ and biotin, which can potentiate the therapeutic efficacy of the compositions. Useful dosages typically will be in the range from about 1 to 10 RDA's (recommended daily allowance) per day.

The invention is further illustrated by reference to the following non-limiting
30 examples.

Effect of Tocotrienyl Lipoates or Tocotrienols and Alpha-Lipoic Acid on Prostaglandin Synthesis as a Measure of Inflammatory Response as a Surrogate For NF-Kappa B Modulated Response.

5 Anti-inflammatory activity of tocotrienyl lipoates or tocotrienols and alpha-lipoic acid materials was conducted using the skin cell line 2[®] ZK1301. The cytotoxicity of the test materials is estimated by the MTT50 of each material (*i.e.*, the concentration required for 50% reduction in the cell viability of treated tissues relative to the untreated controls.)

10 MATERIALS AND EQUIPMENT

- (1) ZK1301 skin 2[®] kit
- (1) ZA0022 MTT Assay Kit
- (1) ZA0080 PGE₂ Assay Kit

Assay kits are available from Advanced Tissue Sciences, Inc.

15

EXPERIMENTAL DESIGN

Tissue Model: skin 2[®] Model ZK 1301

Test Material: 1) T3 as 5.2% rice tocots in 84% PEG 600, 11% non-tocol rice lipids.

20

Rice tocots have a distribution of 47% gamma-tocotrienol, 23% alpha-tocopherol, 22% gamma-tocopherol, 6% delta-tocopherol, 2% alpha-tocotrienol and 1% delta-tocotrienol, other tocotrienol-like compounds may be present.

25

2) T3 and alpha-lipoic acid as 4.9% rice tocots and 4.7% alpha lipoic acid in 80%PEG 600, 11% non-tocol rice lipids.

30

Exposure Mode: Topical application - 2-3 μ l

PMA Concentration: 10 ng/ml, in the media
10 ng/ml, 26 μ l on an applicator pad
PMA Preincubation Time: 24 Hours
Sampling Time: 0, 6 and 24 hours after test material application
5 Replicates: Triplicate per test condition
Assay Media: Serum-Free Assay Media (DMEM)
Control: T3 acetates as 5.0% rice tocots equivalent, 84% PEG
600, 11% non-tocol rice lipids

10 Each test material was tested with and without phorbol myristal acetate ("PMA") pretreatment. PMA stock solution is prepared at 10 μ g/ml in 95% ethanol and stored at 20°C. The PMA is added to the assay media just prior to feeding the tissues.

ASSAY PROCEDURE

15 The calibration and operation of all equipment is checked and documented prior to beginning the study. The assay procedures for 1-[4,5 Dimethylthiazol-2-yl]-3,5-diphenylformazan (MTT) and PGE₂ are as follows. The tissues are removed from the agarose shipping tray and placed in MILLICELL® plates containing serum-free assay media with 10 ng/ml PMA under each MILLICELL®. An applicator pad dosed with
20 25 μ l of PMA is then placed on the epidermal side of the tissues. The tissues are then incubated overnight in a 37°C, 5% CO₂, \geq 90% humidity incubator. The media containing the PMA is aspirated after 24 hours and replaced with fresh serum free assay media without PMA. The tocotrienol, or tocotrienol and alpha lipoic acid, are dispensed directly on the epidermal side of the tissues. Three tissues are dosed for
25 each test material and control. Once all tissues have been dosed, the 0 time control media samples are collected and frozen at -20°C. Fresh serum-free assay media without PMA are added and the plates are placed in a 37°C, 5% CO₂, \geq 90% humidity incubator for the exposure time indicated above. The media are collected at each time point and frozen at -20°C for later analysis for PGE₂. After the last time point,
30 the tissues are placed in a 6-well plate containing MTT and incubated for two hours.

The formazan dye is extracted from the tissues with isopropanol and the optical density is determined at 540 nm.

DATA ANALYSIS

5 The percent untreated control value for all of the dilutions of each test material is calculated as follows:

$$\frac{(\text{Test Material O.D.})}{(\text{Untreated Control O.D.})} \times 100 = \text{Percent Untreated Control}$$

10 The MTT optical density values for the untreated control tissues are assumed to represent 100% viability.

PGE₂ release is measured with a commercially available immunoassay. Concentrations of PGE₂ in the test samples are determined from a standard curve generated with each run. Data is expressed as total pg released per tissue.

15 Prostaglandin E₂ (PGE₂) release is an indicator of a response to PMA challenge involving membrane perturbation, events that activate phospholipase A₂, protein kinase C ("PKC") activation, and the expression of gene products regulated by NF-Kappa B activation. The results of the PGE₂ assay set forth below (Table 1) show a synergistic effect of tocotrienols and alpha-lipoic acid in suppressing synthesis of

20 PGE₂ and that this result was achieved without cytotoxicity (Table 2).

TABLE 1

PGE₂ RELEASE FROM SKIN2 ZK1301 CULTURES CHALLENGED WITH PMA
pg/tissue

Hours	0	1	6
Control PEG	972	1,316	2,381
Control PEG (PMA)	1,411	1,477	3,885
Tocotrienols (PMA)	546	889	2,092
Tocotrienols + Lipoic (PMA)	1,344	355	210

TABLE 2

MTT CELL VIABILITY AFTER 24 HRS INCUBATION
mean MTT o.d. 540 nm.

	Viability-%PEG CONTROL	Std. Deviation
Control PEG	100%	0.03
Control PEG (PMA)	100%	0.05
Tocotrienols (PMA)	134%	0.1
Tocotrienols + Lipoic (PMA)	120%	

SUMMARY

Cell viability was determined using the MTT dye reduction assay. MTT is a substrate for mitochondrial succinate dehydrogenase and is converted to an insoluble formazan by the activity of this enzyme. The amount of formazan produced is proportional to the number of viable cells in the tissue.

At six hours the cell cultures challenged by PMA and protected by the combination of tocotrienols and alpha lipoic acid released 20 times less PGE₂ than the challenged controls, and 10 times less than the cultures protected by tocotrienols alone. This combination is not cytotoxic.

Synthesis of Tocotrienyl lipoate

Tocotrienyl lipoates may be synthesized using dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to facilitate the esterification of alpha-lipoic acid to the tocotrienol or tocopherol in a manner similar to that used by V. Azais-Braesco for the synthesis of Vitamin A esters of fatty acids in JAOCs 69:1272-1273 (1992), incorporated herein by reference.

The following materials were used:

- 1) 32% Tocotrienol and tocopherol concentrate from rice bran oil (RT3) with an average molecular weight ("MW") of the tocol (the combined tocotrienol and tocopherol) from rice bran oil of 420 at 0.72 mmol/gm;
- 2) 98% +/- Alpha tocopherol (AT1) MW 430 at 2.4 mmol/gm;
- 3) 70% mixed tocopherol concentrate from soy & corn oil (MT1) with an average MW of tocol of 420 at 1.7 mmol/gm;
- 4) 98% Alpha-lipoic Acid (ALA) MW 206 at 4.8 mmol/gm;
- 5). 100% Dicyclohexylcarbodiimide (DCC) MW 206, 4.8 mmol/gm;
- 6) 100% Dimethylaminopyridine (DMAP) MW 122, 8.2 mmol/gm; and
- 7) Dichloromethane (MeCl_2), alumina oxide (activated, weakly acidic 150 mesh, (anhydrous)).

Procedures for Synthesis of Tocotrienyl and Tocopheryl lipoates

To reduce potential water or other volatile contaminants, all glassware was cleaned, microwaved until hot, about 1 to 2 minutes, then purged with pure nitrogen to remove traces of water or other solvents. Dichloromethane was purified by passage through a column with Alumina oxide, activated, weakly acidic 150 mesh (anhydrous dry packed).

Synthesis 1: Under low light conditions, solution A was prepared with:

- 1) 5.29 gm of RT3 (3.8 mmol);
- 2) 0.94 gm of ALA (4.3 mmol) [1:1.3 mmol T1 & T3: mmol ALA];
- 3) 0.0547 gm of DMAP (0.45 mmol); and
- 4) 30-35 ml of MeCl_2 .

Solution A was swirled and then sonicated in a bath type sonicator to dissolve all compounds fully. Solution A was then continuously purged with N_2 until solution B was added. Due to this purging the temperature of solution A was about 10-15 degrees C.

5 Solution B was prepared with 0.9-1.1 gm of DCC (approximately 4.8 mmol) and 10 ml of $MeCl_2$. Solution B was swirled to dissolve DCC fully, then it was added to Solution A while swirling in two 5 ml aliquots 2 minutes apart. After all of solution B had been added, the flask was again purged with N_2 , capped with an air tight stopper and placed in the dark overnight.

10 This flask was swirled several more times at irregular time intervals to insure good mixing. Shortly after all of solution B was added, the reaction solution became cloudy with a precipitate that was assumed to be urea.

The reaction solution was filtered through a fast sped paper filter, then a medium speed paper filter, and the filter was washed with 5-10 ml of $MeCl_2$ to recover product. The filtrate was washed sequentially with water, then acid, 0.25 N HCl, then a basic solution of approximately 0.9N Na_2CO_3 , and then water with MeOH in a beaker using a spin bar to provide agitation to remove unwanted compounds. The washed $MeCl_2$ was dried over Na_2SO_4 to remove water, the solvent was removed ($MeCl_2$ evaporated) on a hot water bath with a stream of N_2 . Prior to being fully dry, a precipitate was observed. This was removed by filtration in the same manner as above and solvent evaporation continued. The yellow colored oil product was then analyzed by HPLC.

Synthesis 2: Under low light conditions, solution A was prepared with:

- 1) 5.22 gm of RT3 (3.8 mmol);
25 2) 0.937 gm of ALA (4.3 mmol) [1:1.3 mmol T1 & T3: mmol ALA];
3) 0.054 gm of DMAP (0.45 mmol) and
4) 30-35 ml of $MeCl_2$.

Solution A was swirled then sonicated in a bath type sonicator to dissolve all compounds fully. Solution A was then continuously purged with N_2 until solution B

was added. Due to this purging, the temperature of solution A was about 10-15 degrees C.

Solution B was prepared with 2.06 gm of DCC in 20 ml of MeCl_2 . Solution B was swirled to dissolve DCC fully, then 10.5 ml of B (1.03 gm, approximately 4.8 mmol) was added to Solution A while swirling. After 10.5 ml of solution B had been added, the flask was again purged with N_2 , capped with an air tight stopper and placed in the dark overnight. This flask was swirled several more times at irregular time intervals to ensure good mixing. Shortly after all of solution B was added, the reaction solution became cloudy with a precipitate that was believed to be urea.

The reaction solution was filtered through a fast speed filter, then a medium speed paper filter, and the filter was washed with 5-10 ml of MeCl_2 to recover product. The filtrate was washed sequentially with water, then acid, 0.25 N HCl, then a basic solution of approximately 0.9N Na_2CO_3 , and then water in a beaker using a spin bar to provide agitation to remove unwanted compounds. The washed MeCl_2 was dried over Na_2SO_4 to remove water, the solvent was removed (MeCl_2 evaporated) on a hot water bath with a stream of N_2 . Prior to being fully dry, a precipitate was observed. This was removed by filtration in the same manner as above and solvent evaporation continued. The yellow colored oil product was then analyzed by HPLC.

Saponification: 0.0606 gm of RT3-ALA was placed in a 12 ml screw cap centrifuge tube and saponified in 5 ml of EtOH with 1.5 ml of 40% KOH in MeOH at 50 to 70 degrees C for 15 minutes. After transfer to a 50 ml screw cap centrifuge tube the base was neutralized with 10 ml of 1N HCl and extracted with 10 ml of hexane. The hexane layer was decanted and dried over Na_2SO_4 to remove water then diluted for analysis.

Synthesis 3: Under low light conditions, 0.538 gm of AT1 and 6.188 gm of MT1 were mixed together to make a mixed T1 oil.

Solution A was prepared with:

- 1) 2.064 gm of the foregoing T1 oil (1.75 mmol/gm = 3.61 mmol);
- 2) 0.872 gm of ALA (4.2 mmol)[1:1.17 mmol T1 : mmol ALA];
- 3) 0.052 gm of DMAP (0.43 mmol), and

4) 30-35 ml of MeCl_2 .

Solution A was swirled then sonicated in a bath type sonicator to dissolve all compounds fully. Solution A was then continuously purged with N_2 until solution B was added. Due to this purging, the temperature of solution A was about 10 - 15 degrees C.

Solution B was prepared with 2.06 gm of DCC in 20 ml of MeCl_2 . Solution B was swirled to dissolve DCC fully, then 9.0 ml of solution B (0.881gm, 3.8 mmol) was added to solution A while swirling. After 9 ml of solution B had been added, the flask was again purged with N_2 , capped with an air tight stopper and placed in the dark overnight. This flask was swirled several more times at irregular time intervals to ensure good mixing. Shortly after all of solution B was added, the reaction solution became cloudy with a precipitate that was believed to be urea.

The reaction solution was filtered through a fast speed paper filter, then a medium speed paper filter, and the filter was washed with 5-10 ml of MeCl_2 to recover product. The filtrate was washed sequentially with water, then acid, 0.25 N HCl, then a basic solution of approximately 0.9N Na_2CO_3 , and then water in a beaker using a spin bar to provide agitation to remove unwanted compounds. The washed MeCl_2 was dried over Na_2SO_4 to remove water, the solvent was removed (MeCl_2 evaporated) on a hot water bath with a stream of N_2 . Prior to being fully dry a precipitate was observed. This was removed by filtration in the same manner as above and solvent evaporation continued. The yellow colored oil product was then analyzed by HPLC.

Identification procedure:

Qualitative/ Quantitative analysis of reaction product, starting reactants (tocopherols & tocotrienols) and saponified reaction product (test of ester formation) was carried out via HPLC under the following conditions:

Normal Phase NP

MATERIALS:

MICROSORB CYANO COLUMN : Rainin Instrument Company; F510037;

3 micron Particle Size

4.6 x 100mm Column Size

MOBILE PHASE: Hexane:Isopropanol:Ethyl Acetate/Acetic Acid

HEX:IPA:EA/AA 98.7:0.7:0.3/0.3 @ 1.2ml /min or

Hexane:Isopropanol @ 1.3ml /min

5 MOBILE PHASE: HEX:IPA 99.1 @ 1.3 ml/min.

HEWLETT PACKARD HPLC 1090I WITH 20 or 50 microliter SAMPLE LOOP

HEWLETT PACKARD 1046A FLUORESCENCE DETECTOR: Set at 295/340 nm
Excitation/emission PMT 10 and Diode Array Detector (UV/Vis) collect at 215 nm,
285 nm, 295 nm, 340 nm, 450 nm; spectra collected 250-400nm.

10

Reverse Phase RP

Vydac C18 Column: 218 TP54 250X 4.6 mm, 5 um particle size, Separations Group

MOBILE PHASE: Acetonitrile/Methanol/Ethyl Acetate & Acetic Acid 1:1
95:4.5:0.25/0.25 @1.6 ml/min.

15

Standards ALPHA T-1 STD (Sigma) ALPHA T-1 ACETATE STD (Sigma)

GT-2 70% MIXED STD, TENOX (Eastman SB10-0295)

RT3 32% RICE T1&T3 RANGSIT, RT3-A 32% RICE T1&T3 Acetates RANGSIT

Synthesis 1 and 2 used rice tocols (RT3). It was found that the reaction
products as set forth above contained tocotrienyl lipoates and tocopheryl lipoates.

20

Confirmation that the desired product had been produced can be obtained from the
data collected from the chromatograms and spectra of the parent tocotrienols and
tocopherols and the reaction products. The change in retention times, and shift in
spectral character indicate that a compound other than tocotrienols and tocopherols
were present. The spectral similarity of the tocotrienyl and tocopheryl lipoates to

25

their corresponding acetates (and succinates) in the 260-290 nm range is indicative
of ester formation at the phenolic OH group of tocotrienols and tocopherols. The
lipoates have characteristic absorbance in the 300-400 nm range which are similar
to alpha-lipoic acid. This absorbance is only slightly effected by esterification. There
was virtually no trace of the original tocotrienols and tocopherols in the reaction
products indicating near quantitative synthesis of the desired product. Confirmation

30

of the ester nature of the product was obtained by the regeneration of the parent tocotrienols and tocopherols after saponification, with the concurrent disappearance of the presumed tocotrienyl lipoates and tocopheryl lipoates.

5 The synthesis and conformation of tocopheryl lipoates from natural and synthetic tocopherols was done in Synthesis 3. The data was collected from the chromatograms of the parent tocopherols and the reaction products and spectra of the parent tocopherols and reaction products. The change in retention time, and shift in spectral character indicate that a compound other than tocopherols are present. The spectral similarity of the tocopheryl lipoates to their corresponding acetates (and succinates) in the 260-290 nm range is indicative of ester formation at the phenolic OH group of tocopherols. The lipoates have characteristics in the 300-400 nm range, similar to alpha-lipoic acid. There was virtually no trace of the original tocopherols in the reaction products indicating near quantitative synthesis of the desired product.

10 Individual isomers, *e.g.*, alpha, beta, gamma, delta, epsilon, of tocotrienols and tocopherols or combinations of isomers of tocotrienols and tocopherols may be used in combination with alpha-lipoic acid or esterified with alpha-lipoic acid. Gamma-tocotrienol may preferably be used. The R or S form of alpha-lipoic acid may be used. The R form is preferred.

15 In general other isoprenoids having a phenolic or alcohol group can be synthesized by the method set forth above. Specifically, the following compounds, which it is believed can perform similar functions to tocotrienyl lipoates or tocotrienol and alpha-lipoic acid, can also be used and can be synthesized by the method set forth above. These include terpene alcohol esters or ethers of alpha lipoic acid. Some examples of these esters are: tocotrienyl succinate, linaloyl lipoate made from linanol, Molecule No. 5520 in the 12th Ed. of the Merck Index; farnesyl lipoate, made from farnesol, Molecule No. 3978 in the 12th Ed. of the Merck Index; geranyl lipoate, made from geranol, Molecule No. 4411 in the 12th Ed. of the Merck Index, nerol lipoate made from nerol, Molecule No. 6560 in the 12th Ed. of the Merck Index. Other suitable compounds include gamma-hydroxybutyrate ester or ether of alpha

lipoic acid, cysteine ester of tocopherol and tocotrienol and the succinate ester or ether of tocotrienol.

It is to be understood that the commercial names and sources set forth above are not intended to be limiting and that other commercial designations and/or sources of the same or similar products could be readily determined by those of ordinary skill in the art.

The compositions of the present invention can be administered orally, topically, parenterally, by suppository and by other standard routes of administration. Tocotrienyl lipoate is preferentially absorbed topically as compared to tocotrienol. Combinations of administration routes which yield the greatest bioavailability of a compound or combination of compound can be used. For example, administration of compound 1 by an oral route and administration of compound 2 by a suppository is preferred.

The compositions of the present invention can be administered as an ingredient of along with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers as are known in the art can be used with the present invention.

Compositions for oral administration include capsules, tablets, dispersible powders, granules, syrups, elixirs and suspensions. These compositions can contain one or more conventional adjuvants, such as sweetening agents, flavoring agents, coloring agents and preserving agents.

Tablets can contain the active ingredients in a mixture with conventional pharmaceutically acceptable excipients. These include inert carriers, such as calcium carbonate, sodium carbonate, lactose, and talc; granulating and disintegrating agents, such as starch and alginic acid; binding agents such as starch, gelatin acacia; and lubricating agents, such as magnesium stearate, stearic acid and talc. Tablets may be uncoated or coated by known techniques to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over a longer period of time.

Capsules may contain the active ingredients alone or an admixture with an inert solid carrier, such as calcium carbonate, calcium phosphate or kaolin. Similarly,

suspensions, syrups and elixirs may contain the active ingredients in mixture with any of the conventional excipients utilized in the preparation of such compositions. This includes suspending agents such as methylcellulose, tragacanth and sodium alginate; wetting agents such as lecithin, polyoxyethylene stearate or polyoxyethylene sorbidan
5 monoleate; and preservatives.

The compositions of the present invention are administered in at least such amount that a symptom of a disease, as would be defined by standard medical practice, is improved to the extent that an improvement in the symptom is measurable by a standard test or perceptible to a human with the disease and not in
10 an amount to cause harm to a human or animal.

Alternatively, compositions of the present invention can be administered in at least such amount as to regulate NF-kappa B activity by reducing transcription of a gene product controlled by NF-kappa B by between about 5% and about 99%, preferably between about 25% and about 99%, more preferably between about 50%
15 and about 99%. Reduction of gene transcription can be determined as set forth in Arenzana-Seisdedos, F. et al. Mol. Cell Biol. 15:2689-2696 (1995) and Suzuki et al. Biochem. Biophys. Research Comm. 189:1709-15 (1992), each of which is incorporated herein by reference. Generally, an effective dosage is from about 25 mg to about 2500 mg per day. Dosages may vary from 125 mg to 1250 mg or from 250
20 mg to about 500 mg per day.

Compositions for use in treating smokers

The compositions described above can be used for the treatment or prevention of lung cancers in tobacco smokers. Preferably the compositions also include the following additives in the amounts stated:

25	Vitamin C	5-10 RDAs
	Vitamin B ₆	3-5 RDAs
	biotin	3-5 RDAs

What is claimed is:

1. A method of treating a disease selected from the group consisting of cancer, cancer metastasis, arterial sclerosis, abnormal platelet aggregation, hypertension, congestive lung disease, diabetes, viral infection, septic shock, graft versus host disease, cystic fibrosis, autoimmune disease, neurodegenerative disease, asthma, gout, kidney failure, Parkinson's disease, multiple sclerosis and Alzheimer's disease, comprising the step of administering to a human or an animal in need of such treatment a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to reduce a symptom of said disease.
2. The method of claim 1 wherein said composition is administered in an amount of about 25 mg to about 2500 mg per day.
3. The method of claim 2 wherein said composition is administered in an amount of about 125 mg to about 1250 mg per day.
4. The method of claim 3 wherein said composition is administered in an amount of about 250 mg to about 500 mg per day.
5. The method of claim 2 wherein said composition is administered in an amount of about 1000 mg to about 2500 mg per day.
6. The method of claim 1 wherein said derivatives are selected from the group consisting of linaloyl lipoate, farnesyl lipoate, geranyl lipoate, nerol lipoate, gamma-hydroxybutyrate ester of alpha lipoic acid, gamma-hydroxybutyrate ether of alpha lipoic acid, cysteine ester of tocopherol, cysteine ester of tocotrienol, succinate ester of tocotrienol, and succinate ether of tocotrienol.
7. The method of claim 1 wherein said composition is incorporated into a functional food.
8. The method of claim 1 wherein said composition includes at least one additional nutraceutical or antioxidant.

9. A method of regulating cellular apoptosis comprising the step of administering to a human or an animal in need of such regulation a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to regulate cellular apoptosis.

10. A method of treating a disease caused at least in part by the effect of reactive oxygen species on NF-kappa B, said method comprising the step of administering to a human or an animal in need of such treatment a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof, thereby regulating NF kappa B.

11. The method of claim 10 wherein said composition is administered in an amount effective to reduce transcription of a gene product transcriptionally controlled by NF-kappa B by between about 5% and about 99%.

12. The method of claim 10 wherein said composition is administered in an amount effective to reduce transcription of a gene product transcriptionally controlled by NF-kappa B by between about 25% and about 99%.

13. The method of claim 10 wherein said composition is administered in an amount effective to reduce transcription of a gene product transcriptionally controlled by NF-kappa B by between about 50% and about 99%.

14. The method of claim 11 wherein said gene product is selected from the group consisting of a cytokine, a cytokine receptor, a cell adhesion molecule, a viral protein, a growth factor, a growth factor receptor, an enzyme that produces an anti-oxidant, an immunoreceptor, and a transcription factor.

15. The method of claim 11 wherein said gene product is nitrogen monoxide synthase.

16. The method of claim 10 wherein said composition is administered in an amount effective to reduce a symptom of a disease at least in part caused by a gene product controlled by NF-kappa B.

17. The method of claim 10 wherein said composition is administered orally.
18. The method of claim 17 wherein said composition is incorporated into a functional food.
19. The method of claim 17 wherein said composition includes at least one additional nutraceutical or antioxidant.
20. The method of claim 10 wherein said composition is administered in an amount of about 25 mg to about 2500 mg per day.
21. The method of claim 10 wherein said composition is administered in an amount of about 125 mg to about 1250 mg per day.
22. The method of claim 10 wherein said composition is administered in an amount of about 250 mg to about 500 mg per day.
23. A method of regulating cellular NOx metabolism comprising the step of administering to a human or an animal a composition comprising an ingredient selected from the group consisting of a gamma-tocotrienol, a gamma-tocotrienyl lipoate, a combination of a gamma-tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof.
24. The method of claim 23 wherein regulation of said cellular NOx metabolism comprises regulating the transcription of nitrogen monoxide synthase.
25. The method of claim 23 wherein regulation of said cellular NOx metabolism comprises decreasing peroxynitrite formation.
26. The method of claim 23 wherein said composition is administered in an amount of about 25 mg to about 2500 mg per day.
27. A composition comprising (i) an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof and (ii) a pharmaceutically acceptable carrier, said ingredient formulated in an amount from greater than about 1000 mg to about 2500 mg.

28. A composition comprising (i) an ingredient selected from the group consisting of linaloyl lipoate, farnesyl lipoate, geranyl lipoate, nerol lipoate, gamma-hydroxybutyrate ester of alpha lipoic acid, gamma-hydroxybutyrate ether of alpha lipoic acid, cysteine ester of tocopherol, cysteine ester of tocotrienol, succinate ester of tocotrienol, and succinate ether of tocotrienol and (ii) a pharmaceutically acceptable carrier.

29. A method of preventing a disease selected from the group consisting of cancer, cancer metastasis, arterial sclerosis, abnormal platelet aggregation, hypertension, congestive lung disease, diabetes, viral infection, septic shock, graft versus host disease, cystic fibrosis, autoimmune disease, neurodegenerative disease, asthma, gout, kidney failure, Parkinson's disease, multiple sclerosis and Alzheimer's disease, comprising the step of administering to a human or an animal in need of such treatment a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to prevent occurrence of a symptom of said disease.

30. A method of preventing a disease caused at least in part by the effect of reactive oxygen species on NF-kappa B, said method comprising the step of administering to a human or an animal in need of such treatment a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof, thereby regulating NF kappa B.

31. A method of reducing the occurrence of a disease selected from adenocarcinoma and squamous cell carcinoma in a human who smokes tobacco products and is predisposed to lung cancer by at least one inherited polymorphism selected from the group consisting of a cytochrome P450 1A1 polymorphism and a glutathione S-transferase M1 polymorphism, which comprises the step of administering to said human a composition comprising an effective detoxifying amount of an ingredient selected from the group consisting of a tocotrienyl lipiate, a tocotrienol in combination with alpha-lipoic acid, a derivative thereof, and combinations thereof.

32. The method of claim 31 wherein said composition is administered in an amount of about 5 mg to about 2500 mg per day.

33. The method of claim 31 wherein said derivative is selected from the group consisting of linaloyl lipiate, farnesyl lipiate, geranyl lipiate, nerol lipiate, gamma-hydroxybutyrate ester of alpha lipoic acid, gamma-hydroxybutyrate ether of alpha lipoic acid, cysteine ester of tocopherol, cysteine ester of tocotrienol, succinate ester of tocotrienol, and succinate ether of tocotrienol.

34. A method of reducing the occurrence of a disease selected from the group consisting of adenocarcinoma and squamous cell carcinoma in a human who smokes tobacco products and is predisposed to lung cancer by at least one inherited polymorphism selected from the group consisting of a cytochrome P450 1A1 polymorphism and a glutathione S-transferase M1 polymorphism which comprises the step of administering to said human a composition comprising an effective amount of a component that promotes the intracellular conversion of cystine to cysteine.

35. The method of claim 34 wherein said composition further comprises an effective amount of a components that reduces the intracellular formation of at least one nitrosamine.

36. The method of claim 34 wherein said composition further comprises at least one nutraceutical or antioxidant.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/16207

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/385, 31/35

US CL :514/440, 456, 824, 866, 885, 903

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/440, 456, 824, 866, 885, 903

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chem. abstr., Vol. 124, 1995 (Columbus, OH, USA), the abstract No. 54478, TOMEO, A.C. 'Antioxidant Effects of Tocotrienols In Patients With Hyperlipidemia And Carotid Stenosis.' Lipids 1995, 30(12), 1479-1483.	1-9, 27-29, 31-36
A	Chem. abst., Vol. 111, 1989 (Columbus, OH, USA), the abstract No. 70438, KOMIYAMA, K. 'Studies On The Biological Activity Of Tocotrienols.' Chem. Pharm. Bull. 1989, 37(5), 1369-1371.	1-9, 27-29, 31-36
A	US 5,591,772 A (LANE et al.) 07 January 1997.	1-9, 27-29, 31-36
A	US 5,348,974 A (WRIGHT et al.) 20 September 1994.	1-9, 27-29, 31-36

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
O Document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

24 OCTOBER 1998

Date of mailing of the international search report

09 NOV 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

KIMBERLY JORDAN

Telephone No. (703) 308-1235

International application No.
PCT/US98/16207

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chem. abstr., Vol. 125, 1996 (Columbus, OH, USA), the abstract No. 237967, MERIN, J.P. 'alpha-Lipoic Acid Blocks HIV-1 LTR-Dependent Expression Of Hygromycin Resistance In THP-1 Stable Transformants.' FEBS Lett. 1996, 394(1), 9-13.	10-22, 30
A	Chem. abstr., Vol. 120, 1993 (Columbus, OH, USA), the abstract No. 6622, PACKER, L. 'Vitamin E And alpha-Lipoate: Role In Antioxidant Recycling And Activation Of The NF-kappa-B Transcription Factor.' Mol. Aspects Med. 1993, 14(3), 229-239.	10-22, 30
A	Chem. abstr., Vol. 120, 1994 (Columbus, OH, USA), the abstract No. 124631, FAUST, A. 'Effect Of Lipoic Acid On Cyclophosphamide-Induced Diabetes And Insulitis In Non-Obese Diabetic Mice.' Int. J. Immunopharmacol. 1994, 16(1), 61-6.	23-26

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS AND CAS ONLINE: lipoate? or lipoic or tocotrienyl or tocotrienol with cancer? or carcinoma or neoplast? or arteriosclero? or atherosclero? or platelet or hypertens? or diabet? or autoimmune or asthma or gout or Parkinson? or multiple sclerosis or Alzheimer? or nf-kappa? or nitric oxide





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/385, 31/35	A1	(11) International Publication Number: WO 99/06040 (43) International Publication Date: 11 February 1999 (11.02.99)
(21) International Application Number: PCT/US98/16207 (22) International Filing Date: 4 August 1998 (04.08.98) (30) Priority Data: 60/055,433 4 August 1997 (04.08.97) US (71)(72) Applicants and Inventors: BERRY, Christopher, J. [NZ/TH]; 72/1 Soi Sailom, Phaholyothin Road (Soi 8), Samsennai, Phyathi, Bangkok 10400 (TH). FOLEY, John, L. [US/US]; 31 Campus Plaza Road, Hadley, MA 01035 (US). PACKER, Lester [US/US]; 64 Charles Hill Road, Orinda, CA 94563 (US). (74) Agents: BERMAN, Rod, S. et al.; Jeffer, Mangels, Butler & Marmaro LLP, 10th floor, 2121 Avenue of the Stars, Los Angeles, CA 90067-5010 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHOD OF TREATING DISEASE USING A TOCOTRIENOL AND ALPHA-LIPOIC ACID OR DERIVATIVES OR AN ESTER THEREOF (57) Abstract Antioxidant or combination of antioxidants which function particularly well in modulating the action of free radicals and in particular which function particularly well in regulating activation of a transcription factor, such as NF-kappa B and which can be non-toxically administered to a human or an animal are disclosed. In particular a method of treating diseases, including reduction of the incidence of lung cancers in tobacco smokers, using a tocotrienol and alpha-lipoic acid or tocotrienyl lipoate or derivatives thereof is disclosed.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

5 The present invention relates to a method of treating diseases, more specifically using a combination of a tocotrienol and alpha-lipoic acid or using a tocotrienyl lipoate or using derivatives thereof. The present invention further relates, in particular to a method of reducing the occurrence of the excess incidence of cardiovascular disease and specific types of cancer in tobacco smokers.

Many diseases have been found to be caused in whole or in part by the detrimental action of free radicals. In order to treat such diseases the administration of a compound, referred to as an antioxidant, which counteracts the action of these free radicals, has been described. Such administration is disclosed by the following:

15 United States Patent No. 5,348,974 and United States Patent No. 5,217,992 disclose the use of tocotrienols to treat hypercholesterolemia, hyperlipidemia and thromboembolic disorders; United States Patent No. 5,376,361 discloses the use of tocotrienols and derivatives and tocopherols and tocopherol esters for topical administration to the skin to prevent and/or treat radiation-induced skin damage;

20 United States Patent No. 5,204,373 discloses the use of carba-tocotrienols as having superior metabolic stability over tocotrienols; United States Patent No. 4,603,142 discloses the use of a specific species of tocotrienol to lower cholesterol; United States Patent No. 5,334,612 discloses the treatment of retroviral diseases, such as HIV, using alpha-lipoic acid; United States Patent No. 5,084,481 discloses the

25 treatment of pain and inflammatory disorders, including diabetic neuropathy and arteriosclerosis, using dihydrolipoic acid.

Additionally, it has been found that many diseases caused in whole or in part by free radicals involve the expression of gene products which are under the control of the nuclear transcription factors known as NF-kappa B and AP-1. NF-kappa B and

- 2 -

AP-1 regulation in turn is modulated by the free radical status of a cell which can activate translocation of NF-kappa B from the cell cytosol to the cell nucleus where it can bind to DNA and facilitate transcription of gene products involved in disease states. Antioxidants, by affecting the free radical status of the cell, can regulate the activation of NF-kappa B and AP-1.

A progressive deterioration in the antioxidant network characterizes the aging process. As a result of this deterioration, intracellular free radical levels rise. This in turn changes the regulation of the transcription of NF-kappa B and AP-1 related genes. More specifically, there is an age-associated alteration in nuclear factor kappa-B and AP-1 signal transduction pathways. See, e.g., Helenius et al., *Biochem. J.*, 318 (Pt. 2) ():603-8 (Sept 1, 1996) (NF-kappa B); Wortwein et al., *J. Neurosci. Res.* 52(3):322-33 (May 1, 1998) (AP-1). AP-1 and NF-kappa B both show increased activities in older animals, whereas most other transcription factors decrease in DNA binding activities with age. Sierra et al., *J. Gerontol. A. Biol. Sci. Med. Sci.* 53(2):B102-B110 (Mar. 1998). Similar levels of NF-kappa B were found in the cytosol of T-cells from both young and elderly donors. The precursor levels of NF-kappa B remain unaltered during aging.

The age-associated decline is caused by altered regulation of the inhibitor I-kappa B, the redox-mediated activation step. Trebilcock et al., *Gerontology* 42(3):137-146 (1996). A functional antioxidant network assessed in terms of glutathione levels is required for NF-kappa B activation. Ginn-Pease et al., *Biochem. Biophys. Res. Commun.* 226(3):695-702 (Sept. 24, 1996). Glutathione levels decline with age and disease.

The transcription of gamma-glutamylcysteine synthetase is up-regulated by TNF-alpha. This up-regulation is attenuated by preincubation with high physiological levels of glucose. Thus, high glucose levels impair the regulation of glutathione. See, e.g., Morales et al., *J. Biol. Chem.* 272(48):30371-9 (Nov. 28, 1997). Glutathione peroxidase overexpression in turn down-regulates thresholds of NF-kappa B activation by altering the isoform composition of I-kappa B. See, e.g., Suyang et al., *Mol. Cell.*

Biol. 16(10):5444-9 (Oct. 1996); Kretz-Remy et al., J.Cell. Biol. 133(5):1083-93 (June 1996).

Cancers are among the diseases in which the action of free radicals has been implicated. Numerous other agents are known to cause various types of cancers.

5 Among the various cancers, lung cancer is the most common cause of cancer deaths in men and women in the United States and worldwide. The two major histopathologies of lung cancer, adenocarcinoma (AC) and squamous carcinoma (SCC), affect smoker particularly.. AC was very rare 30 years ago and not thought to be associated with tobacco use. However, the incidence of AC has risen 10 to 20
10 times in the last generation, and it now accounts for almost as many lung cancer cases as SCC. See, e.g., Stellman et al., Prev. Med. 26(4):451-6 (July-Aug. 1997); Wynder et al., Environ. Health Perspect., 103 Suppl 8():143-8 (Nov. 1995).

Squamous carcinoma (SCC) is thought to be caused by polynuclear aromatic hydrocarbons (PAHs) in tars. It occurs in the entrances to the air passageways in the
15 lung, whereas AC is promoted by gas phase components of cigarette smoke and occurs deep inside the lung tissue.

The huge increase in the rate of incidence of AC is thought to result from changes in cigarette manufacturing techniques. See Hoffman, J. Toxicol. Environ. Health 50(4):307-64 (March 1997). Since the 1950s, tobacco companies have
20 altered the way in which cigarettes are formulated. Tar and nicotine yields have declined from a high of 38 milligrams of tar and 2.7 milligrams of nicotine in 1954 to 12 milligrams of tar and 0.95 milligrams of nicotine today. These reductions in tar content have been achieved by introducing filters and by adding nitrates to tobacco. Average nitrate levels have increased from 0.4% to just over 1%. These nitrates
25 cause the cigarette to burn hotter, decreasing tar yields, but at the expense of increased generation of nitrogenous compounds, which are the gaseous carcinogens implicated in the rise of adenocarcinomas. By adding nitrates to tobacco, cigarette companies have reduced the tar yields which they are obliged to disclose but introduced a new hidden killer which apparently is responsible for a twenty-fold
30 increase in the rate of adenocarcinomas.

- 4 -

PAHs, the main carcinogen in tobacco tar, are detoxified in two stages by human cells. First, cytochrome P450-related compounds break down the PAHs into a form which can be solubilized by the enzyme glutathione S-transferase. Glutathione S-transferase bonds the PAH residues to glutathione, preparing them to be excreted in the water phase.

5 Glutathione is the major antioxidant system in the body, and is synthesized in cells by enzymes from three dietary amino acids: glutamate, cysteine and glycine. Glutathione-related enzymes are encoded by a superfamily of genes. See, e.g., Lafuente et al., *Cancer Lett.* 68 (1):49-54 (Jan. 15, 1993). These genes include GST
10 M1 and GST T1. Almost half of the population inherits less than optimal glutathione-related genes. The GST M1 gene is deleted in 50% of the population and the GST T1 gene in 16%. Hayes et al., *Crit. Rev. Biochem. Mol. Biol.* 20(6):445-600 (1995). Thus, as aging occurs, or as the functionality of the antioxidant network is
15 impaired by infection, carcinogens or metabolic stresses, or inherited suboptimal genetic polymorphisms, the function of the transcription factors NF-kappa B and AP-1, is attenuated. See, e.g., Pinkus et al., *J. Biol. Chem.* 271(23):13422-9 (June 7, 1996). It has been suggested that these genetic abnormalities are major
20 determinants of susceptibility to degenerative diseases such as cancers, for example tobacco-related lung cancers, and many studies have found elevated risks. Further, as the normal production of cytokines is disturbed, there is dysregulation of the
hypothalamic/pituitary/adrenal (HPA) axis, resulting in hypertension, diabetes, arterial sclerosis and heart failure. Inherited variations in the genes which regulate
antioxidant enzyme transcription exacerbate these conditions. Subjects inheriting
25 polymorphisms of the cytochrome P450 and glutathione transferase gene families are at elevated risk of degenerative disease.

Recently researchers at Ninewells Hospital and Medical School in Dundee, Scotland, bred a strain of mice in which the gene that encodes glutathione S-transferase was knocked out. See *Proc. Nat'l Acad. Sci. USA* 95(9):5275-80 (April 28, 1998). This genetic structure mimics the abnormality found in 46% of

- 5 -

Caucasians. These mice were shown to be three times more likely to develop carcinomas as a result of PAH exposure.

Early human studies (e.g., Grinberg-Funes et al., *Carcinogenesis* 15(11):2449-54 (Nov. 1994) found elevated risks of lung cancer in humans who inherit the structure, but not all of these studies have shown high increased risk of carcinoma development. Recently, several studies have reported that weaknesses in the glutathione-related genetic mechanisms characterize early-onset lung cancer. See, e.g., el-Zein et al, *Mutat. Res.* 381(2):189-200 (Nov. 28, 1997); Bouchardy et al., *Cancer Res.* 56(2):251-3 (Jan. 15, 1996); Kihara et al., *Carcinogenesis* 16(10):2331-6 (Oct. 1995); Anttila et al., *Cancer Res.* 55(15):3305-9 (Aug. 1, 1995); Nakajima et al., *Carcinogenesis* 16(4):707-11 (Apr. 1995); Kihara et al., *Carcinogenesis* 15(2):415-8 (Feb. 1994); Kawajiri et al., *Princess Takamatsu Symp.* 21(): 55-61 (1990).

These studies show an interplay between total pack-years of smoking, glutathione S-transferase knockout and lung cancer. In people with compromised glutathione S-transferase genes, lung cancer risk elevates dramatically with increased total exposure to tobacco smoke. It appears that susceptibility to lung cancer results from the interplay between the inherited capacity of the detoxification mechanisms and lifetime exposure to tobacco smoke carcinogens. Prolonged smoking exhausts the detoxification system. In people inheriting a weak genetic detoxification structure this occurs more quickly.

Furthermore, cigarette smokers are at elevated risk of stroke, hypertension and cardiovascular disease. Approximately half of the premature death among smokers is from heart attacks and strokes. Cigarette smokers have elevated cortisol levels (see, e.g., Pomerleau et al., *Pharmacol. Biochem. Behav.* 36(1):211-3 (May 1990)), and whole blood from smokers has been found to have a 38% higher TNF production after lipopolysaccharide stimulation. Tappia et al., *Clin. Sci. (Colch.)* 88(4):485-9 (April 1995).

Often the mechanisms by which cigarette smoking exacerbates these conditions are related to dysregulation of the HPA axis, which modulates the interplay between

the hormones which regulate glucose metabolism (insulin), and those that counter-regulate glucose metabolism (glucocorticoids). Epinephrine and norepinephrine mediate short-term adjustments, whereas cortisol influences longer term cyclic responses.

5 The apparent insulin resistance and hypertension which characterize cigarette smokers, Syndrome X, adult onset diabetes and Cushing's Syndrome, share a common antecedent in disturbances to the HPA axis. Insulin resistance can be considered as dysregulation of and/or an increased tissue sensitivity to glucocorticoids. Thioredoxin, a redox-regulating cellular cofactor that modulates
10 cellular glucocorticoid responsiveness, influences tissue sensitivity to glucocorticoids. See, e.g., Makino et al., J. Clin. Invest. 98(11):2469-77 (Dec. 1, 1996).

 The HPA axis is a complex open system, modulated by counter-regulatory hormones, by inhibitory feedback loops and by cytokines. Cytokines modulate glucocorticoid hormones and are modulated by them. Since cellular production of
15 cytokines is controlled by transcription factors, principally NF-kappa B, and NF-kappa B activation is controlled by cellular redox status, antioxidants modulate the HPA axis.

 The interaction between glucocorticoids and insulin is mediated through thresholds of NF-kappa B expression. NF-kappa B and the glucocorticoid receptor physically interact, resulting in the repression of NF-kappa B transactivation.
20 Glucocorticoids inhibit cytokine gene transcription through receptor cross-coupling. The activated glucocorticoid receptor can bind to and inactivate AP-1 and NF-kappa B.

 Glucocorticoids strongly downregulate TNF-alpha. Pretreatment of cells with glucocorticoids modulates NF-kappa B activation, TNF-alpha and serum glucose.
25 Administration of glucocorticoids alters hormonal and cytokine response to lipopolysaccharide in humans. Elevated TNF and cortisol levels have been found to exaggerate S-TNF-R responses for up to 144 hours after infusion, and in some tissue glucocorticoids upregulate the transcription of I-kappa B isoforms which bind to NF-kappa B in the cytosol and modulate NF-kappa B mediated gene transcription.
30 Glucocorticoids thus are the link between insulin resistance and hypertension.

The HPA system, as a whole, is in dynamic semi-stable equilibrium and pivots around set points analogous to thermostats. At birth, these set points are predetermined by the environment of the womb, when the fetus is exposed to the maternal regulatory system. Consequently, it has been demonstrated that low birth weight children have higher cortisol levels as adults, much higher incidence of adult-onset diabetes, and hypertension, stroke and heart disease as adults. Subsequently, contemporary stresses such as nocturnal hypoglycemia, disrupted sleep patterns, psychological stress, cigarette smoke, environmental toxins or aging, can disrupt the setpoints and influence HPA function by affecting NF-kappa B activation thresholds.

The onset of degenerative disease in middle age is frequently foreshadowed by cumulative changes in HPA responses consequent to exhaustion of the antioxidant network measured by decreasing glutathione levels, increasing TNF alpha levels, and a lowered threshold of NF-kappa B activation, increasing blood pressure, impairment of the diurnal regulation of cortisol, increased tissue sensitivity to glucocorticoids and apparent insulin resistance.

Excess cortisol has profound effects on the metabolism of glucose and protein. It increases gluconeogenesis by increasing glycogenolysis in muscle to provide lactate as a gluconeogenic precursor. It increases lipolysis in the periphery to provide glycerol as a gluconeogenic precursor. Since cortisol has anti-insulin effects, it decreases the uptake of glucose into muscle, adipose, lymphoid and fibroblast tissues. Excess cortisol also breaks down muscle proteins to provide gluconeogenic precursors to the liver. Disregulation of these effects lead to hyperglycemia and a characteristic pattern of central obesity.

The programming of the HPA axis has been shown to influence body weight, blood pressure body fat disposition patterns, insulin resistance and depression. Diabetics have significantly greater 24-hour urinary-free cortisol output which increases with duration of diabetes and degree of diabetic complications. Diabetic neuropathy is associated with specific and persistent increases in HPA axis activity. Cortisol levels are elevated in symptomatic polyneuropathy.

Cytokines whose expression is controlled by NF-kappa B, particularly TNF-alpha, the cytokine implicated in insulin resistance, modulate the HPA axis and certain of the metabolic consequences of cortisol elevation are modulated by redox sensitive steps.

5 Glucocorticoids also have mineralocorticoid activities. Excess glucocorticoids may spill over onto aldosterone receptors to increase renal Na⁺ absorption and increase renal K⁺ and H⁺ excretion, retaining water in the ECF, and elevating blood pressure. Also, glucocorticoids synergize catecholamines, increasing stroke volume and cardiac output which can also cause hypertension.

10 Cortisol levels rise in response to nicotine in smokers and are permanently elevated by cortisol-secreting tumors in Cushing's Syndrome, while in adult onset diabetes the diurnal cycle of cortisol secretion is disturbed and cortisol responses to TNF are exaggerated. Inappropriately elevated cortisol levels affect renin/angiotensin receptors inducing sodium retention and hypertension, counter-regulate insulin and
15 attenuate insulin-mediated uptake of glucose by GLUT-4 receptors in muscle tissue. Cigarette smoke condensate activates NF-kappa B, directly compounding the disregulation. See Shen et al., Am.J.Physiol. 270(5 Pt.2):H1624-33 (May 1996).

 Thus, the disruption of normal HPA axis function caused when nicotine raises cortisol levels and ROS overwhelm the antioxidant network contributes substantially
20 to the elevated cardiovascular risk of smokers. One of the consequences of nicotine elevating cortisol levels in smokers is that cortisol, which normally responds to low blood glucose levels in order to protect the brain from hypoglycemia, is instead elevated by a false nicotine-mediated signal and insulin down-regulated when blood glucose levels are still high, resulting in the area under the curve (AUC) of blood
25 glucose rising and further impairing the transcription of protective antioxidant enzymes. Urata et al., J. Biol. Chem. 271(25):15146-52 (June 21, 1996). Consequently, glycation and the formation of advanced glycation end products (AGEs) is elevated, raising cardiovascular risks by mechanisms similar to those by which the cardiovascular risks of diabetics are raised. AGE and AGE receptor (RAGE)

interactions activate NF-kappa B, as does glycated LDL, and activated NF-kappa B is found in arteriosclerotic lesions.

AGE-apolipoprotein B and serum AGE levels in cigarette smokers have been found to be significantly higher than those in non-smokers. See Cerami et al.,
5 "Tobacco Smoke is a Source of Toxic Reactive Glycation Products," The Picower Institute for Medical Research, Manhasset, NY 11030 USA (1997). Cigarette smoke is a source of reactive AGE compounds. Id. It is therefore not surprising that diabetics who smoke have 2-4 times the incidence of cardiovascular disease than non-smoking diabetics.

10 The HPA is an open, adaptive system whose components interact in complex, self-regulating redundant patterns. The effects of isolated components can be paradoxical and shifted in time. For example, severely elevated cortisol levels can produce depression or euphoria, and normal responses to stimuli can be inverted or attenuated in diabetics and hypertensives or after prolonged oxidative stress.

15 The cellular processes which control the gene transcription of cytokines depend on the integrity of the antioxidant signaling network. In many degenerative conditions, including the tobacco-related diseases, it is the attenuation of the antioxidant signaling network by increasing reactive oxygen species' stress loads that trigger disease onset. Redox sensitive pathways modulate the HPA axis directly, by
20 controlling the transcription of cytokine messengers, and indirectly by ameliorating the consequences of HPA dysfunction by reducing glycation damage. Thus, strengthening and reinforcing this network can delay the onset of degenerative diseases.

Accordingly, a need exists for an antioxidant or combination of antioxidants
25 which function particularly well in maintaining the integrity of the HPA axis and protecting against the elevated cardiovascular risk reflected in impaired glucose tolerance, increased formation of advanced glycation end products and hypertension.

A need also exists for an antioxidant or a combination of antioxidants which function particularly well in modulating the action of free radicals and in particular

which function particularly well in regulating activation of NF-kappa B and which can be non-toxically administered to a human or an animal.

A need also exists for a method of treating diseases, such as cancer, in which the action of free radicals has been implicated.

5 There is also a need for a method of reducing the occurrence of cancers in human patients who are especially susceptible to such occurrence. In particular, a need exists for a method of reducing the occurrence of lung cancer in human patients who smoke tobacco products and who are predisposed to lung cancer by inherited polymorphisms in cytochrome P450 1A1 and glutathione S-transferase M, such as
10 those described, e.g., in Lafuente et al., Cancer Lett. 68(1):49-54 (Jan. 15, 1993); Kihara et al., Carcinogenesis 16(10):2331-6 (Oct. 1995); Grinberg-Funes et al., Carcinogenesis 15(11):2449-54 (Nov. 1994); and Bouchardy et al., Cancer Res. 56(2):251-3 (Jan. 15, 1996).

Summary of the Preferred Embodiments

15 In accordance with one aspect of the present invention, there are provided methods of treating and preventing a disease selected from the group consisting of cancer, arterial sclerosis, abnormal platelet aggregation, hypertension, congestive lung disease, diabetes, viral infection, septic shock, graft versus host disease, cystic fibrosis, autoimmune disease, neurodegenerative disease, asthma, gout, kidney
20 failure, Parkinson's disease, multiple sclerosis, and Alzheimer's disease. The methods comprise the step of administering to a human or an animal, preferably a mammal, in need of such treatment a composition including an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to
25 reduce or prevent a symptom of such disease.

 In accordance with another aspect of the present invention, there is provided a method of regulating cellular apoptosis. The method comprises the step of administering to a human or an animal a composition including an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and

alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to regulate cellular apoptosis.

According to another aspect of the present invention there are provided methods of treating and preventing a disease caused at least in part by the effect of reactive oxygen species on NF-kappa B. The method comprise the step of administering to a human or an animal, preferably a mammal, in need of such treatment a composition including an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof, thereby regulating NF-kappa B.

In a preferred embodiment, the composition is administered in an amount effective to reduce transcription of a gene product controlled by NF-kappa B by between about 5% and about 99%.

Preferably the gene product is selected from the group consisting of a cytokine, a cytokine receptor, a cell adhesion molecule, a viral protein, a growth factor, a growth factor receptor, an immunoreceptor, a transcription factor, an oncogene and nitric acid synthase.

In accordance with another aspect of the present invention, there is provided a method of regulating cellular NO_x metabolism comprising the step of administering to a human or an animal a composition including an ingredient selected from the group consisting of a gamma-tocotrienol, a gamma-tocotrienyl lipoate, a combination of a gamma-tocotrienol with alpha-lipoic acid, derivatives thereof, such as other isomers of vitamin E that are "free" at the ortho position on the phenolic moiety, and combinations thereof.

In accordance with another aspect of the present invention there is provided a method of improving the regulation of the HPA axis-mediated interplay between cortisol and insulin and of protecting the cardiovascular system from the sequelae of its disturbance, hypertension, diminished HDLC levels, and increased advanced glycation end products.

In a first preferred embodiment, the method of regulating cellular NO_x metabolism comprises regulating nitric oxide synthase activity.

- 12 -

In a second preferred embodiment, the method of regulating cellular NO_x metabolism comprises decreasing the formation of peroxynitrites.

In yet another aspect of the present invention there is provided a composition including an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof, and a pharmaceutically acceptable carrier. Preferably the composition is formulated in an amount from greater than about 1000 mg to about 2500 mg.

Preferably the foregoing derivatives are selected from the group consisting of linaloyl lipoate, farnesyl lipoate, geranyl lipoate, nerol lipoate, gamma-hydroxybutyrate ester of alpha lipoic acid, gamma-hydroxybutyrate ether of alpha lipoic acid, cysteine ester of tocopherol, cysteine ester of tocotrienol, succinate ester of tocotrienol, and succinate ether of tocotrienol. The derivatives are preferably administered in an amount from about 25 mg to about 1000 mg.

In accordance with still another aspect of the present invention, there is provided a method of reducing the occurrence of a disease selected from the group consisting of adenocarcinoma and squamous cell carcinoma in a human who smokes tobacco products and is predisposed to lung cancer by at least one inherited polymorphism selected from the group consisting of a cytochrome P450 1A1 polymorphism and a glutathione S-transferase M1 polymorphism, which comprises the step of administering to the human a composition including an effective detoxifying amount of an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof.

In accordance with still another aspect of the present invention, there is provided a method of reducing the occurrence of a disease selected from the group consisting of adenocarcinoma and squamous cell carcinoma in a human who smokes tobacco products and is predisposed to lung cancer by at least one inherited polymorphism selected from the group consisting of a cytochrome P450 1A1 polymorphism and a glutathione S-transferase M1 polymorphism which comprises the

- 13 -

step of administering to the human a composition including an effective amount of a component that promotes the intracellular conversion of cystine to cysteine.

In a preferred embodiment, the composition further includes an effective amount of a components that reduces the intracellular formation of at least one nitrosamine.

Other objects, features and advantages of the present invention will become apparent to those skilled in the art from the following detailed description. It is to be understood, however, that the detailed description and specific example, while indicating preferred embodiments of the present invention, are given by way of illustration and not limitation. Many changes and modifications within the scope of the present invention can be made without departing from the spirit thereof, and the invention includes all such modifications.

Detailed Description of the Preferred Embodiments

It has been discovered that tocotrienyl lipoate or a combination of a tocotrienol and alpha-lipoic acid act synergistically as compared to a tocotrienol alone or alpha-lipoic acid alone in reducing or blocking inflammatory responses and in regulating the activity of transcription factors sensitive to a cell redox state, such as the DNA transcription factor NF-kappa B.

NF-kappa B is part of a family of Rel transcription factors that share common characteristics. They consist of hetero- or homodimeric proteins in association with an inhibitory protein family, I-kappa B. It is believed that one mechanism by which NF-kappa B is activated is by phosphorylation and dissociation of I-kappa B from Rel protein dimers, followed by I-kappa B's proteolytic degradation, which allows the dimeric DNA-binding protein NF-kappa B to enter the nucleus and regulate gene transcription. A host of genes have been shown to be modulated by NF-kappa B, including genes for cytokines and growth factors, immunoreceptors, adhesion molecules, acute-phase proteins, transcription factors and regulators, NO-synthase, and viral genes. A general description of the NF-kappa B transcriptional regulation system can be found in Bauerele and Baltimore, *Cell*, Volume 87, 13-20 (1996), incorporated herein by reference.

- 14 -

Oxygen free radicals and nitrogen monoxide and their by-products that are capable of causing oxidative damage are collectively referred to as active or reactive oxygen species ("ROS") and reactive nitrogen species ("RNS"). These compounds are also known as "free radicals." These ROS can activate NF-kappa B and it is believed that ROS are the final common signal for a number of stimuli that activate NF-kappa B. Sen and Packer, *The FASEB Journal*, Vol. 10, 709-720 (1996). The administration of antioxidants which can modulate the ROS status of a cell should therefor be useful in regulating NF-kappa B activation. The activation of NF-kappa B is believed to be involved, at least in part, in the causation or progression of a number of disease states. Packer et al., *Advances in Pharmacology*, Vol. 38, 79-101 (1997).

For example, acquired immunodeficiency syndrome results from infection with a human immunodeficiency virus (HIV-1 or HIV-2), which eventually destroys a specific subset (CD4 +) of helper T-lymphocytes, so that the patient ultimately yields to opportunistic infection and certain neoplasms. The long terminal repeat (LTR) region of HIV-1 proviral DNA contains two binding sites for NF-kappa B, which activate transcription by binding to the sequence 5'-GGGACTTTCC-3' in the kappa enhancer where it interacts with the transcription apparatus. In this case, and for other viruses as well, the virus usurps normal cellular machinery in order to transcribe its own DNA.

By blocking activation of NF-kappa B, interference with the viral reproductive cycle can be facilitated.

Oxidants appear to be involved in two steps of the atherosclerotic process. Low density lipoprotein ("LDL") particles enter the artery wall and some remain. In this environment the LDL becomes mildly oxidized, possibly by ROS or RNS released by vascular cells. The mildly oxidized LDL contains a component or components, believed to be oxidized phospholipid, which causes the endothelium to secrete molecules that result in the recruitment of monocytes and their differentiation to macrophages. Further oxidation of the LDL results in its uptake by macrophages, their conversion to foam cells, and deposition in the vascular wall as part of fatty-

streak lesions. Maziere et al., *Biochem. and Molec. Bio. Intl.*, Vol. 39(6) pp. 1201-1207 (1996).

5 NF-kappa B appears to be a central transcription factor in the expression of a number of the genes induced by the early oxidation step in atherosclerosis, including serum amyloid A and macrophage colony-stimulating factor. When C57BL/6J mice and C3H/HeJ mice, which are susceptible and not susceptible to formation of aortic fatty streaks, respectively, are fed an atherogenic diet, only the C57BL/6J mice show activation of NF-kappa B.

10 There is a further acceleration of arterial sclerosis in diabetes and in cigarette smokers, which is consequent to prolonged elevation of blood glucose levels. This is hypothesized to be due to advanced glycosylation end products (AGE) interacting with their endothelial receptor to induce the expression of vascular cell adhesion molecule-1 (VCAM-1), an early feature in the pathogenesis of atherosclerosis. In cultured human endothelial cells, exposure to AGEs induced expression of VCAM-1
15 associated with increased levels of VCAM-1 transcripts; electrophoretic mobility shift assays indicated that there was also induction of specific DNA-binding activity for NF-kappa B in the VCAM-1 promoter, which was blocked by N-acetyl cysteine ("NAC"). Other studies also indicate that the induction of expression of VCAM-1 in response to a variety of signals is mediated by an NF-kappa B-like DNA-binding protein and is
20 blocked by a variety of antioxidants, including pyrrolidine dithiocarbamate and NAC.

Accelerated arterial sclerosis in diabetes and cigarette smokers may be due to the presence of larger amounts of AGE, a greater degree of interaction of these with the receptor for AGE (AGE-RAGE interaction), activation of NF-kappa B, expression of VCAM-1, and priming of the diabetic vasculature for enhanced interaction with
25 circulating monocytes.

By interfering with the activation of NF-kappa B, these diseases, and other diseases which are believed to be caused at least in part by the effect of reactive oxygen species on NF-kappa B (for example by NF-kappa B activation of gene transcription) can be treated. The following diseases in addition to the above-listed
30 are believed to be so caused: cancer, cancer metastasis, abnormal platelet

- 16 -

aggregation, hypertension, congestive lung disease, viral infection, such as HIV/AIDS, septic shock, graft versus host disease, cystic fibrosis, autoimmune disease, neurodegenerative disease, asthma, gout, kidney failure, Parkinson's disease, multiple sclerosis, and Alzheimer's disease, as well as cellular apoptosis. With respect to cellular apoptosis, regulation of NF-kappa B with the compositions of the present invention can cause or inhibit apoptosis, depending upon the particular cell type and the redox state of the cell, thereby regulating cellular apoptosis. To the extent that an animal is susceptible to disease states caused by NF-kappa B activation, such animal may be similarly treated.

10 A disease may be considered treated by the inventive methods if a symptom of the disease is improved to the extent that an improvement in the symptom is measurable by a standard test or perceptible to a human with the disease. For example, an increase in CD4+ T-cell levels as measured by standard assay, in a patient with AIDS. See, A Manual of Laboratory and Diagnostic Tests, Fifth Ed. Francis Fishbach (1996), incorporated herein by reference.

15 A disease may be considered prevented by the inventive methods if a disregulation or imbalance leading to the condition is corrected.

While not being bound by a particular theory, it is believed that the synergistic effect of tocotrienols and alpha-lipoic acid is the result of the phytyl side chain of tocotrienols which provides enhanced lipophilic mobility and intermembrane transferability to the tocotrienols. Also, tocotrienols are distributed evenly throughout the biomembrane whereas alpha tocopherol is clumped in relatively immobile clusters. Accordingly, tocotrienols are more readily available to more cellular compartments to interact with free radicals in those compartments. Further, after oxidation of a tocotrienol by its interaction with a free radical the tocotrienol may be more readily reduced by alpha-lipoic acid and thereby able to again interact with a free radical than less mobile antioxidants. The importance of this molecular mobility in ultimately regulating NF-kappa B activity was not heretofore recognized. Further, while some antioxidants can be administered directly to cell cultures in vitro in an amount that will provide a concentration sufficient to affect NF-kappa B activity, the compositions

- 17 -

of the present invention, particularly tocotrienols and alpha lipoic acid, unlike the afore-mentioned antioxidants, can be preferably orally administered or administered by another route to a human or an animal in an amount to provide a concentration in vivo sufficient to modulate NF-kappa B activity.

5 The regulation of cellular nitrogen monoxide ("NO") and nitrogen dioxide ("NO₂") metabolism ("NO_x") is important with respect to carcinogenesis, particularly carcinogenesis caused by exposure to environmental air pollutants. NO₂ can form peroxynitrites which are potent free radicals and can damage cells by inducing lipid peroxidation and protein oxidation. Peroxynitrites can also nitrosate amines; nitrosated amines can cause DNA mutations, which can lead to carcinogenesis. NO_x radicals can also activate NF-kappa B which in turn can activate transcription of inducible nitrogen monoxide synthase.

10 Inducible nitrogen monoxide synthase is long lasting, Ca²⁺ independent and glucocorticoid sensitive. Nitrogen monoxide synthase generates NO leading to a positive feedback cycle of inducible nitrogen monoxide synthase production. In contrast, constitutive nitrogen monoxide synthase activity is short lasting, having one sixteenth to one tenth the activity of inducible nitrogen monoxide synthase, is Ca²⁺ dependent and is hormone insensitive. It has been found that gamma-tocotrienols due to the absence of a methyl group at the C-5 position can form a C-nitroso aromatic compound which thereby decreases the production of detrimental peroxynitrites, thereby regulating "NO_x" metabolism. This effect can be augmented by administering or esterifying alpha-lipoic acid with the gamma-tocotrienol, due to the ability of the alpha-lipoic to regenerate oxidized tocotrienol radicals to tocotrienols. Gamma-tocotrienols are believed to preferentially operate by the same mechanisms as set forth above. Therefore administration of gamma-tocotrienols can reduce free radical induced DNA mutagenesis and resulting carcinogenesis.

25 It has further been discovered that the administration of alpha lipoic acid and tocotrienols in a compound or mixture according to the present invention is effective in treating cancers, in particular lung cancer, both therapeutically and prophylactically. That is, it has been discovered that such administration is effective

30

SUBSTITUTE SHEET (RULE 26)

in protecting against both the AC and SCC forms of lung cancer associated with cigarette smoking. Such compositions, in particular gamma-tocotrienol lipolate, are especially effective in maintaining glutathione-related detoxification mechanisms in cigarette smokers who have inherited sub-optimal combinations of the cytochrome P450 and glutathione gene superfamily. Further, the gamma-isomer of tocotrienol appears to offer additional protection against nitrosamine-induced adenocarcinomas.

Han et al., Biofactors 6(3):321-38 (1997) have shown that the rate limiting step in the cellular synthesis of glutathione is the availability of cysteine in the cytosol. Glutathione is the most important antioxidant/detoxification pathway in the human body. Depressed glutathione levels are an indicator of mortality in many disease models. Yet glutathione levels are notoriously difficult to boost. Packer et al. showed that administration of alpha-lipoic acid to cell cultures elevated glutathione levels by 30% by splitting cystine to cysteine, thereby facilitating the entry of cysteine into the cell by a channel that is 10 times faster than the cystine channel. Since the final step in the detoxification of PAHs requires the enzyme glutathione S-transferase to join glutathione to the toxicant, boosting or restoring intracellular glutathione levels indirectly through alpha-lipoic administration, may help protect those smokers with an inherited attenuation of the glutathione system which detoxifies PAHs, thereby reducing their vulnerability to squamous carcinomas. Since the epidemiological pattern indicates that squamous carcinomas occur after the detoxification system has been exhausted by long-term exposure to cigarette smoke, it follows that administration of alpha-lipoic may have a very significant role in boosting glutathione-related detoxification mechanisms thereby postponing or preventing the incidence of squamous carcinomas.

Tocotrienols have been reported to have various anti-cancer effects including the modulation of glutathione S-transferase levels in animal cancer models. In particular, gamma-tocotrienol is 40 to 60 times more readily recycled in membranes than alpha-tocopherol, and the gamma-isomers of tocopherols and tocotrienols appear to function to detoxify nitrogen oxides present in cigarette smoke by protecting against the formation of carcinogenic nitrosamines. Furthermore, alpha-lipoic acid

- 19 -

appears to recycle tocotrienols. The effects of tocotrienols and alpha-lipoic have been shown to be synergized when co-administered.

Remarkably, each of these compounds synergizes the other within the domain of its respective protective action. The addition of tocotrienols to alpha-lipoic acid potentiates the modulation by alpha-lipoic acid of the glutathione-related pathways and reinforces the effectiveness of cytochrome P450, while the addition of alpha-lipoic acid to tocotrienols facilitates the systemic recycling of tocotrienols, thereby facilitating detoxification of nitrogen metabolites. The compensatory modulation of gene expression achieved by this pairing is unique and previously unrecognized.

Preferably, an effective amount of the inventive composition for use in preventing the occurrence of lung cancers as discussed herein is about 5 mg to about 2500 mg, more preferably about 100 mg to about 1250 mg. Preferably the molar ratio of alpha-lipoic acid to tocotrienol is about 1:100 to about 2:1. The R-form of alpha-lipoic acid is particularly potent. Advantageously about 2-10 mg of the R-form (2-10 times the usual dietary intake) are employed to synergize with tocotrienol. Naturally occurring R-alpha lipoic acid is frequently found in lipamide form.

Desirably, the inventive compositions described herein are administered in divided doses 30-45 minutes prior to meals. However, other modes of administration of the inventive compositions will also be readily apparent to those skilled in the art. For example, the inventive compositions can be incorporated into a functional food for consumption by a patient.

According to the invention, a reduction in the *in vivo* glutathione level of about 5-30% is realized, resulting in effective reduction in the occurrence of AC and SCC in tobacco smokers who are predisposed to lung cancer by virtue of an inherited polymorphism in cytochrome P450 1A1 and/or glutathione S-transferase M1.

The inventive compositions can also include additional antioxidants and/or nutraceuticals, such as vitamin C, vitamin B₆ and biotin, which can potentiate the therapeutic efficacy of the compositions. Useful dosages typically will be in the range from about 1 to 10 RDA's (recommended daily allowance) per day.

- 20 -

The invention is further illustrated by reference to the following non-limiting examples.

Effect of Tocotrienyl Lipoates or Tocotrienols and Alpha-Lipoic Acid on Prostaglandin Synthesis as a Measure of Inflammatory Response as a Surrogate For NF-Kappa B Modulated Response.

5 Anti-inflammatory activity of tocotrienyl lipoates or tocotrienols and alpha-lipoic acid materials was conducted using the skin cell line 2^o ZK1301. The cytotoxicity of the test materials is estimated by the MTT50 of each material (i.e., the concentration required for 50% reduction in the cell viability of treated tissues relative
10 to the untreated controls.)

MATERIALS AND EQUIPMENT

- (1) ZK1301 skin 2^o kit
- (1) ZA0022 MTT Assay Kit
- 15 • (1) ZA0080 PGE₂ Assay Kit

Assay kits are available from Advanced Tissue Sciences, Inc.

EXPERIMENTAL DESIGN

20 Tissue Model: skin 2^o Model ZK 1301
Test Material: 1) T3 as 5.2% rice tocals in 84% PEG 600, 11% non-tocol rice lipids.
Rice tocals have a distribution of 47% gamma-tocotrienol, 23% alpha-tocopherol, 22% gamma
25 tocopherol, 6% delta-tocopherol, 2% alpha-tocotrienol and 1% delta-tocotrienol, other tocotrienol-like compounds may be present.
2) T3 and alpha-lipoic acid as 4.9% rice tocals and 4.7% alpha lipoic acid in 80%PEG 600, 11% non-tocol rice
30 lipids.

- 21 -

Exposure Mode: Topical application - 2-3 μ l
PMA Concentration: 10 ng/ml, in the media
10 ng/ml, 26 μ l on an applicator pad
PMA Preincubation Time: 24 Hours
5 Sampling Time: 0, 6 and 24 hours after test material application
Replicates: Triplicate per test condition
Assay Media: Serum-Free Assay Media (DMEM)
Control: T3 acetates as 5.0% rice tocols equivalent, 84% PEG
600, 11% non-tocol rice lipids

10

Each test material was tested with and without phorbol myristal acetate ("PMA") pretreatment. PMA stock solution is prepared at 10 μ g/ml in 95% ethanol and stored at 20°C. The PMA is added to the assay media just prior to feeding the tissues.

15

ASSAY PROCEDURE

The calibration and operation of all equipment is checked and documented prior to beginning the study. The assay procedures for 1-[4,5 Dimethylthiazol-2-yl]-3,5-diphenylformazan (MTT) and PGE₂ are as follows. The tissues are removed from the agarose shipping tray and placed in MILLICELL® plates containing serum-free assay media with 10 ng/ml PMA under each MILLICELL®. An applicator pad dosed with 25 μ l of PMA is then placed on the epidermal side of the tissues. The tissues are then incubated overnight in a 37°C, 5% CO₂, \geq 90% humidity incubator. The media containing the PMA is aspirated after 24 hours and replaced with fresh serum free assay media without PMA. The tocotrienol, or tocotrienol and alpha lipoic acid, are 25 dispensed directly on the epidermal side of the tissues. Three tissues are dosed for each test material and control. Once all tissues have been dosed, the 0 time control media samples are collected and frozen at -20°C. Fresh serum-free assay media without PMA are added and the plates are placed in a 37°C, 5% CO₂, \geq 90% humidity incubator for the exposure time indicated above. The media are collected at each 30 time point and frozen at -20°C for later analysis for PGE₂. After the last time point,

- 22 -

the tissues are placed in a 6-well plate containing MTT and incubated for two hours. The formazan dye is extracted from the tissues with isopropanol and the optical density is determined at 540 nm.

DATA ANALYSIS

- 5 The percent untreated control value for all of the dilutions of each test material is calculated as follows:

$$\frac{(\text{Test Material O.D.})}{(\text{Untreated Control O.D.})} \times 100 = \text{Percent Untreated Control}$$

10

The MTT optical density values for the untreated control tissues are assumed to represent 100% viability.

- 15 PGE₂ release is measured with a commercially available immunoassay. Concentrations of PGE₂ in the test samples are determined from a standard curve generated with each run. Data is expressed as total pg released per tissue. Prostaglandin E₂ (PGE₂) release is an indicator of a response to PMA challenge involving membrane perturbation, events that activate phospholipase A₂, protein kinase C ("PKC") activation, and the expression of gene products regulated by NF-Kappa B activation. The results of the PGE₂ assay set forth below (Table 1) show a synergistic effect of tocotrienols and alpha-lipoic acid in suppressing synthesis of
- 20 PGE₂ and that this result was achieved without cytotoxicity (Table 2).

- 23 -

TABLE 1

PGE₂ RELEASE FROM SKIN2 ZK1301 CULTURES CHALLENGED WITH PMA
pg/tissue

5	Hours	0	1	6
	Control PEG	972	1,316	2,381
	Control PEG (PMA)	1,411	1,477	3,885
	Tocotrienols (PMA)	546	889	2,092
10	Tocotrienols + Lipoic (PMA)	1,344	355	210

TABLE 2

MTT CELL VIABILITY AFTER 24 HRS INCUBATION
mean MTT o.d. 540 nm.

15		Viability-%PEG CONTROL	Std. Deviation
	Control PEG	100%	0.03
	Control PEG (PMA)	100%	0.05
	Tocotrienols (PMA)	134%	0.1
20	Tocotrienols + Lipoic (PMA)	120%	

SUMMARY

25 Cell viability was determined using the MTT dye reduction assay. MTT is a substrate for mitochondrial succinate dehydrogenase and is converted to an insoluble formazan by the activity of this enzyme. The amount of formazan produced is proportional to the number of viable cells in the tissue.

30 At six hours the cell cultures challenged by PMA and protected by the combination of tocotrienols and alpha lipoic acid released 20 times less PGE₂ than the challenged controls, and 10 times less than the cultures protected by tocotrienols alone. This combination is not cytotoxic.

Synthesis of Tocotrienyl lipoate

Tocotrienyl lipoates may be synthesized using dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to facilitate the esterification of alpha-lipoic acid to the tocotrienol or tocopherol in a manner similar to that used by V. Azais-Braesco for the synthesis of Vitamin A esters of fatty acids in JAOCS 69:1272-1273 (1992), incorporated herein by reference.

The following materials were used:

- 1) 32% Tocotrienol and tocopherol concentrate from rice bran oil (RT3) with an average molecular weight ("MW") of the tocol (the combined tocotrienol and tocopherol) from rice bran oil of 420 at 0.72 mmol/gm;
- 2) 98% +/- Alpha tocopherol (AT1) MW 430 at 2.4 mmol/gm;
- 3) 70% mixed tocopherol concentrate from soy & corn oil (MT1) with an average MW of tocol of 420 at 1.7 mmol/gm;
- 4) 98% Alpha-lipoic Acid (ALA) MW 206 at 4.8 mmol/gm;
- 5). 100% Dicyclohexylcarbodiimide (DCC) MW 206, 4.8 mmol/gm;
- 6) 100% Dimethylaminopyridine (DMAP) MW 122, 8.2 mmol/gm; and
- 7) Dichloromethane (MeCl₂), alumina oxide (activated, weakly acidic 150 mesh, (anhydrous)).

Procedures for Synthesis of Tocotrienyl and Tocopheryl lipoates

To reduce potential water or other volatile contaminants, all glassware was cleaned, microwaved until hot, about 1 to 2 minutes, then purged with pure nitrogen to remove traces of water or other solvents. Dichloromethane was purified by passage through a column with Alumina oxide, activated, weakly acidic 150 mesh (anhydrous dry packed).

Synthesis 1: Under low light conditions, solution A was prepared with:

- 1) 5.29 gm of RT3 (3.8 mmol);
- 2) 0.94 gm of ALA (4.3 mmol) [1:1.3 mmol T1 & T3: mmol ALA];
- 3) 0.0547 gm of DMAP (0.45 mmol); and
- 4) 30-35 ml of MeCl₂.

- 25 -

Solution A was swirled and then sonicated in a bath type sonicator to dissolve all compounds fully. Solution A was then continuously purged with N₂ until solution B was added. Due to this purging the temperature of solution A was about 10-15 degrees C.

5 Solution B was prepared with 0.9-1.1 gm of DCC (approximately 4.8 mmol) and 10 ml of MeCl₂. Solution B was swirled to dissolve DCC fully, then it was added to Solution A while swirling in two 5 ml aliquots 2 minutes apart. After all of solution B had been added, the flask was again purged with N₂, capped with an air tight stopper and placed in the dark overnight.

10 This flask was swirled several more times at irregular time intervals to insure good mixing. Shortly after all of solution B was added, the reaction solution became cloudy with a precipitate that was assumed to be urea.

15 The reaction solution was filtered through a fast sped paper filter, then a medium speed paper filter, and the filter was washed with 5-10 ml of MeCl₂ to recover product. The filtrate was washed sequentially with water, then acid, 0.25 N HCl, then a basic solution of approximately 0.9N Na₂CO₃, and then water with MeOH in a beaker using a spin bar to provide agitation to remove unwanted compounds. The washed MeCl₂ was dried over Na₂SO₄ to remove water, the solvent was removed (MeCl₂ evaporated) on a hot water bath with a stream of N₂. Prior to
20 being fully dry, a precipitate was observed. This was removed by filtration in the same manner as above and solvent evaporation continued. The yellow colored oil product was then analyzed by HPLC.

Synthesis 2: Under low light conditions, solution A was prepared with:

- 25 1) 5.22 gm of RT3 (3.8 mmol);
2) 0.937 gm of ALA (4.3 mmol) [1:1.3 mmol T1 & T3: mmol ALA];
3) 0.054 gm of DMAP (0.45 mmol) and
4) 30-35 ml of MeCl₂.

Solution A was swirled then sonicated in a bath type sonicator to dissolve all compounds fully. Solution A was then continuously purged with N₂ until solution B

- 26 -

was added. Due to this purging, the temperature of solution A was about 10-15 degrees C.

Solution B was prepared with 2.06 gm of DCC in 20 ml of MeCl_2 . Solution B was swirled to dissolve DCC fully, then 10.5 ml of B (1.03 gm, approximately 4.8 mmol) was added to Solution A while swirling. After 10.5 ml of solution B had been added, the flask was again purged with N_2 , capped with an air tight stopper and placed in the dark overnight. This flask was swirled several more times at irregular time intervals to ensure good mixing. Shortly after all of solution B was added, the reaction solution became cloudy with a precipitate that was believed to be urea.

The reaction solution was filtered through a fast speed filter, then a medium speed paper filter, and the filter was washed with 5-10 ml of MeCl_2 to recover product. The filtrate was washed sequentially with water, then acid, 0.25 N HCl, then a basic solution of approximately 0.9N Na_2CO_3 , and then water in a beaker using a spin bar to provide agitation to remove unwanted compounds. The washed MeCl_2 was dried over Na_2SO_4 to remove water, the solvent was removed (MeCl_2 evaporated) on a hot water bath with a stream of N_2 . Prior to being fully dry, a precipitate was observed. This was removed by filtration in the same manner as above and solvent evaporation continued. The yellow colored oil product was then analyzed by HPLC.

Saponification: 0.0606 gm of RT3-ALA was placed in a 12 ml screw cap centrifuge tube and saponified in 5 ml of EtOH with 1.5 ml of 40% KOH in MeOH at 50 to 70 degrees C for 15 minutes. After transfer to a 50 ml screw cap centrifuge tube the base was neutralized with 10 ml of 1N HCl and extracted with 10 ml of hexane. The hexane layer was decanted and dried over Na_2SO_4 to remove water then diluted for analysis.

Synthesis 3: Under low light conditions, 0.538 gm of AT1 and 6.188 gm of MT1 were mixed together to make a mixed T1 oil.

Solution A was prepared with:

- 1) 2.064 gm of the foregoing T1 oil (1.75 mmol/gm = 3.61 mmol);
- 2) 0.872 gm of ALA (4.2 mmol) (1:1.17 mmol T1 : mmol ALA);
- 3) 0.052 gm of DMAP (0.43 mmol), and

- 27 -

4) 30-35 ml of MeCl_2 .

Solution A was swirled then sonicated in a bath type sonicator to dissolve all compounds fully. Solution A was then continuously purged with N_2 until solution B was added. Due to this purging, the temperature of solution A was about 10 - 15 degrees C.

Solution B was prepared with 2.06 gm of DCC in 20 ml of MeCl_2 . Solution B was swirled to dissolve DCC fully, then 9.0 ml of solution B (0.881gm, 3.8 mmol) was added to solution A while swirling. After 9 ml of solution B had been added, the flask was again purged with N_2 , capped with an air tight stopper and placed in the dark overnight. This flask was swirled several more times at irregular time intervals to ensure good mixing. Shortly after all of solution B was added, the reaction solution became cloudy with a precipitate that was believed to be urea.

The reaction solution was filtered through a fast speed paper filter, then a medium speed paper filter, and the filter was washed with 5-10 ml of MeCl_2 to recover product. The filtrate was washed sequentially with water, then acid, 0.25 N HCl, then a basic solution of approximately 0.9N Na_2CO_3 , and then water in a beaker using a spin bar to provide agitation to remove unwanted compounds. The washed MeCl_2 was dried over Na_2SO_4 to remove water, the solvent was removed (MeCl_2 evaporated) on a hot water bath with a stream of N_2 . Prior to being fully dry a precipitate was observed. This was removed by filtration in the same manner as above and solvent evaporation continued. The yellow colored oil product was then analyzed by HPLC.

Identification procedure:

Qualitative/ Quantitative analysis of reaction product, starting reactants (tocopherols & tocotrienols) and saponified reaction product (test of ester formation) was carried out via HPLC under the following conditions:

Normal Phase NP

MATERIALS:

MICROSORB CYANO COLUMN : Rainin Instrument Company; F510037;

3 micron Particle Size

- 28 -

4.6 x 100mm Column Size

MOBILE PHASE: Hexane:Isopropanol:Ethyl Acetate/Acetic Acid

HEX:IPA:EA/AA 98.7:0.7:0.3/0.3 @ 1.2ml /min or

Hexane/Isopropanol @ 1.3ml /min

5 MOBILE PHASE: HEX:IPA 99.1 @ 1.3 ml/min.

HEWLETT PACKARD HPLC 1090I WITH 20 or 50 microliter SAMPLE LOOP

HEWLETT PACKARD 1046A FLUORESCENCE DETECTOR: Set at 295/340 nm
Excitation/emission PMT 10 and Diode Array Detector (UV/Vis) collect at 215 nm,
285 nm, 295 nm, 340 nm, 450 nm; spectra collected 250-400nm.

10

Reverse Phase RP

Vydac C18 Column: 218 TP54 250X 4.6 mm, 5 um particle size, Separations Group

MOBILE PHASE: Acetonitrile/Methanol/Ethyl Acetate & Acetic Acid 1:1
95:4.5:0.25/0.25 @1.6 ml/min.

15 Standards ALPHA T-1 STD (Sigma) ALPHA T-1 ACETATE STD (Sigma)

GT-2 70% MIXED STD, TENOX (Eastman SB10-0295)

RT3 32% RICE T1&T3 RANGSIT, RT3-A 32% RICE T1&T3 Acetates RANGSIT

Synthesis 1 and 2 used rice tocols (RT3). It was found that the reaction
products as set forth above contained tocotrienyl lipoates and tocopheryl lipoates.

20

Confirmation that the desired product had been produced can be obtained from the
data collected from the chromatograms and spectra of the parent tocotrienols and
tocopherols and the reaction products. The change in retention times, and shift in
spectral character indicate that a compound other than tocotrienols and tocopherols

25

were present. The spectral similarity of the tocotrienyl and tocopheryl lipoates to
their corresponding acetates (and succinates) in the 260-290 nm range is indicative
of ester formation at the phenolic OH group of tocotrienols and tocopherols. The

30

lipoates have characteristic absorbance in the 300-400 nm range which are similar
to alpha-lipoic acid. This absorbance is only slightly effected by esterification. There
was virtually no trace of the original tocotrienols and tocopherols in the reaction
products indicating near quantitative synthesis of the desired product. Confirmation

of the ester nature of the product was obtained by the regeneration of the parent tocotrienols and tocopherols after saponification, with the concurrent disappearance of the presumed tocotrienyl lipoates and tocopheryl lipoates.

5 The synthesis and conformation of tocopheryl lipoates from natural and synthetic tocopherols was done in Synthesis 3. The data was collected from the chromatograms of the parent tocopherols and the reaction products and spectra of the parent tocopherols and reaction products. The change in retention time, and shift in spectral character indicate that a compound other than tocopherols are present. The spectral similarity of the tocopheryl lipoates to their corresponding acetates (and
10 succinates) in the 260-290 nm range is indicative of ester formation at the phenolic OH group of tocopherols. The lipoates have characteristics in the 300-400 nm range, similar to alpha-lipoic acid. There was virtually no trace of the original tocopherols in the reaction products indicating near quantitative synthesis of the desired product.

Individual isomers, *e.g.*, alpha, beta, gamma, delta, epsilon, of tocotrienols and
15 tocopherols or combinations of isomers of tocotrienols and tocopherols may be used in combination with alpha-lipoic acid or esterified with alpha-lipoic acid. Gamma-tocotrienol may preferably be used. The R or S form of alpha-lipoic acid may be used. The R form is preferred.

In general other isoprenoids having a phenolic or alcohol group can be
20 synthesized by the method set forth above. Specifically, the following compounds, which it is believed can perform similar functions to tocotrienyl lipoates or tocotrienol and alpha-lipoic acid, can also be used and can be synthesized by the method set forth above. These include terpene alcohol esters or ethers of alpha lipoic acid. Some examples of these esters are: tocotrienyl succinate, linaloyl lipoate made from
25 linanol, Molecule No. 5520 in the 12th Ed. of the Merck Index; farnesyl lipoate, made from farnesol, Molecule No. 3978 in the 12th Ed. of the Merck Index; geranyl lipoate, made from geranol, Molecule No. 4411 in the 12th Ed. of the Merck Index, nerol lipoate made from nerol, Molecule No. 6560 in the 12th Ed. of the Merck Index. Other suitable compounds include gamma-hydroxybutyrate ester or ether of alpha

- 30 -

lipoic acid, cysteine ester of tocopherol and tocotrienol and the succinate ester or ether of tocotrienol.

It is to be understood that the commercial names and sources set forth above are not intended to be limiting and that other commercial designations and/or sources of the same or similar products could be readily determined by those of ordinary skill in the art.

The compositions of the present invention can be administered orally, topically, parenterally, by suppository and by other standard routes of administration. Tocotrienyl lipoate is preferentially absorbed topically as compared to tocotrienol. Combinations of administration routes which yield the greatest bioavailability of a compound or combination of compound can be used. For example, administration of compound 1 by an oral route and administration of compound 2 by a suppository is preferred.

The compositions of the present invention can be administered as an ingredient of along with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers as are known in the art can be used with the present invention.

Compositions for oral administration include capsules, tablets, dispersible powders, granules, syrups, elixirs and suspensions. These compositions can contain one or more conventional adjuvants, such as sweetening agents, flavoring agents, coloring agents and preserving agents.

Tablets can contain the active ingredients in a mixture with conventional pharmaceutically acceptable excipients. These include inert carriers, such as calcium carbonate, sodium carbonate, lactose, and talc; granulating and disintegrating agents, such as starch and alginic acid; binding agents such as starch, gelatin acacia; and lubricating agents, such as magnesium stearate, stearic acid and talc. Tablets may be uncoated or coated by known techniques to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over a longer period of time.

Capsules may contain the active ingredients alone or an admixture with an inert solid carrier, such as calcium carbonate, calcium phosphate or kaolin. Similarly,

- 31 -

suspensions, syrups and elixirs may contain the active ingredients in mixture with any of the conventional excipients utilized in the preparation of such compositions. This includes suspending agents such as methylcellulose, tragacanth and sodium alginate; wetting agents such as lecithin, polyoxyethylene stearate or polyoxyethylene sorbitan monoleate; and preservatives.

The compositions of the present invention are administered in at least such amount that a symptom of a disease, as would be defined by standard medical practice, is improved to the extent that an improvement in the symptom is measurable by a standard test or perceptible to a human with the disease and not in an amount to cause harm to a human or animal.

Alternatively, compositions of the present invention can be administered in at least such amount as to regulate NF-kappa B activity by reducing transcription of a gene product controlled by NF-kappa B by between about 5% and about 99%, preferably between about 25% and about 99%, more preferably between about 50% and about 99%. Reduction of gene transcription can be determined as set forth in Arenzana-Seisdedos, F. et al. Mol. Cell Biol. 15:2689-2696 (1995) and Suzuki et al. Biochem. Biophys. Research Comm. 189:1709-15 (1992), each of which is incorporated herein by reference. Generally, an effective dosage is from about 25 mg to about 2500 mg per day. Dosages may vary from 125 mg to 1250 mg or from 250 mg to about 500 mg per day.

Compositions for use in treating smokers

The compositions described above can be used for the treatment or prevention of lung cancers in tobacco smokers. Preferably the compositions also include the following additives in the amounts stated:

25	Vitamin C	5-10 RDAs
	Vitamin B ₆	3-5 RDAs
	biotin	3-5 RDSs

What is claimed is:

1. A method of treating a disease selected from the group consisting of cancer, cancer metastasis, arterial sclerosis, abnormal platelet aggregation, hypertension, congestive lung disease, diabetes, viral infection, septic shock, graft versus host disease, cystic fibrosis, autoimmune disease, neurodegenerative disease, asthma, gout, kidney failure, Parkinson's disease, multiple sclerosis and Alzheimer's disease, comprising the step of administering to a human or an animal in need of such treatment a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to reduce a symptom of said disease.
2. The method of claim 1 wherein said composition is administered in an amount of about 25 mg to about 2500 mg per day.
3. The method of claim 2 wherein said composition is administered in an amount of about 125 mg to about 1250 mg per day.
4. The method of claim 3 wherein said composition is administered in an amount of about 250 mg to about 500 mg per day.
5. The method of claim 2 wherein said composition is administered in an amount of about 1000 mg to about 2500 mg per day.
6. The method of claim 1 wherein said derivatives are selected from the group consisting of linaloyl lipoate, farnesyl lipoate, geranyl lipoate, nerol lipoate, gamma-hydroxybutyrate ester of alpha lipoic acid, gamma-hydroxybutyrate ether of alpha lipoic acid, cysteine ester of tocopherol, cysteine ester of tocotrienol, succinate ester of tocotrienol, and succinate ether of tocotrienol.
7. The method of claim 1 wherein said composition is incorporated into a functional food.
8. The method of claim 1 wherein said composition includes at least one additional nutraceutical or antioxidant.

- 33 -

9. A method of regulating cellular apoptosis comprising the step of administering to a human or an animal in need of such regulation a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to regulate cellular apoptosis.
10. A method of treating a disease caused at least in part by the effect of reactive oxygen species on NF-kappa B, said method comprising the step of administering to a human or an animal in need of such treatment a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof, thereby regulating NF kappa B.
11. The method of claim 10 wherein said composition is administered in an amount effective to reduce transcription of a gene product transcriptionally controlled by NF-kappa B by between about 5% and about 99%.
12. The method of claim 10 wherein said composition is administered in an amount effective to reduce transcription of a gene product transcriptionally controlled by NF-kappa B by between about 25% and about 99%.
13. The method of claim 10 wherein said composition is administered in an amount effective to reduce transcription of a gene product transcriptionally controlled by NF-kappa B by between about 50% and about 99%.
14. The method of claim 11 wherein said gene product is selected from the group consisting of a cytokine, a cytokine receptor, a cell adhesion molecule, a viral protein, a growth factor, a growth factor receptor, an enzyme that produces an anti-oxidant, an immunoreceptor, and a transcription factor.
15. The method of claim 11 wherein said gene product is nitrogen monoxide synthase.
16. The method of claim 10 wherein said composition is administered in an amount effective to reduce a symptom of a disease at least in part caused by a gene product controlled by NF-kappa B.

- 34 -

17. The method of claim 10 wherein said composition is administered orally.
18. The method of claim 17 wherein said composition is incorporated into a functional food.
19. The method of claim 17 wherein said composition includes at least one additional nutraceutical or antioxidant.
20. The method of claim 10 wherein said composition is administered in an amount of about 25 mg to about 2500 mg per day.
21. The method of claim 10 wherein said composition is administered in an amount of about 125 mg to about 1250 mg per day.
22. The method of claim 10 wherein said composition is administered in an amount of about 250 mg to about 500 mg per day.
23. A method of regulating cellular NO_x metabolism comprising the step of administering to a human or an animal a composition comprising an ingredient selected from the group consisting of a gamma-tocotrienol, a gamma-tocotrienyl lipoate, a combination of a gamma-tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof.
24. The method of claim 23 wherein regulation of said cellular NO_x metabolism comprises regulating the transcription of nitrogen monoxide synthase.
25. The method of claim 23 wherein regulation of said cellular NO_x metabolism comprises decreasing peroxynitrite formation.
26. The method of claim 23 wherein said composition is administered in an amount of about 25 mg to about 2500 mg per day.
27. A composition comprising (i) an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof and (ii) a pharmaceutically acceptable carrier, said ingredient formulated in an amount from greater than about 1000 mg to about 2500 mg.

- 35 -

28. A composition comprising (i) an ingredient selected from the group consisting of linaloyl lipoate, farnesyl lipoate, geranyl lipoate, nerol lipoate, gamma-hydroxybutyrate ester of alpha lipoic acid, gamma-hydroxybutyrate ether of alpha lipoic acid, cysteine ester of tocopherol, cysteine ester of tocotrienol, succinate ester of tocotrienol, and succinate ether of tocotrienol and (ii) a pharmaceutically acceptable carrier.

29. A method of preventing a disease selected from the group consisting of cancer, cancer metastasis, arterial sclerosis, abnormal platelet aggregation, hypertension, congestive lung disease, diabetes, viral infection, septic shock, graft versus host disease, cystic fibrosis, autoimmune disease, neurodegenerative disease, asthma, gout, kidney failure, Parkinson's disease, multiple sclerosis and Alzheimer's disease, comprising the step of administering to a human or an animal in need of such treatment a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a tocotrienol in combination with alpha-lipoic acid, derivatives thereof, and combinations thereof in an amount effective to prevent occurrence of a symptom of said disease.

30. A method of preventing a disease caused at least in part by the effect of reactive oxygen species on NF-kappa B, said method comprising the step of administering to a human or an animal in need of such treatment a composition comprising an ingredient selected from the group consisting of a tocotrienyl lipoate, a combination of a tocotrienol and alpha-lipoic acid, derivatives thereof, and combinations thereof, thereby regulating NF kappa B.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16207

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chem. abstr., Vol. 125, 1996 (Columbus, OH, USA), the abstract No. 237967, MERIN, J.P. 'alpha.-Lipoic Acid Blocks HIV-1 LTR-Dependent Expression Of Hygromycin Resistance In THP-1 Stable Transformants.' FEBS Lett. 1996, 394(1), 9-13.	10-22, 30
A	Chem. abstr., Vol. 120, 1993 (Columbus, OH, USA), the abstract No. 6622, PACKER, L. 'Vitamin E And alpha-Lipoate: Role In Antioxidant Recycling And Activation Of The NF-kappa-B Transcription Factor.' Mol. Aspects Med. 1993, 14(3), 229-239.	10-22, 30
A	Chem. abstr., Vol. 120, 1994 (Columbus, OH, USA), the abstract No. 124631, FAUST, A. 'Effect Of Lipoic Acid On Cyclophosphamide-Induced Diabetes And Insulitis In Non-Obese Diabetic Mice.' Int. J. Immunopharmacol. 1994, 16(1), 61-6.	23-26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/16207

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS AND CAS ONLINE: lipoate? or lipoic or tocotrienyl or tocotrienol with cancer? or carcinoma or neoplast? or arteriosclero? or atherosclero? or platelet or hypertens? or diabet? or autoimmune or asthma or gout or Parkinson? or multiple sclerosis or Alzheimer? or nf-kappa? or nitric oxide